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(54) Title: BIOMATERIAL SUBSTRATES

(57) Abstract: The invention concerns the use of a biomaterial substrate to orient cell or tissue growth. The substrate comprises a base portion and a surface layer covering at least part of the base portion. The surface layer provides the substrate with topographical features having at least one nanoscale dimension of from about 1 to about 200 nm. The topographical features having the capacity to orient cell or tissue growth thereon and/or therebetween.

DESCRIPTION

BIOMATERIAL SUBSTRATES

The present invention relates to novel biomaterial substrates, to methods of making them and to uses therefor. In particular the invention concerns such materials, methods and uses for the orientation of cell or tissue growth.

The properties of cells are influenced by their external environment, both chemical and physical. Understanding interactions which take place between a cell and its substrate is important in connection with such fields as medical implants and prostheses, tissue engineering and pharmaceutical development. One substrate characteristic which has been shown to influence cellular properties is topography and synthetic structured surfaces have been used to investigate this influence. A review of such investigations may be found in *Biomaterials (1999), 20, 573-588.*

The modification of surface topography for the control of cellular response is an important area of research in medical engineering that targets several potential end uses, particularly relating to the biocompatibility of materials used in medical devices. In this area, it is required to control the interfacial reactions that mitigate the appropriate response for a specific application. It is known that the interfacial reactions are influenced by the surface properties of the biomaterial substrate in terms of the surface chemistry, energy and topography. Of the latter, current research is focused on etching techniques to create the desired topography. *Experimental Cell Research (1996), 223, 426-435*, discusses the production of microfabricated grooves and steps by means of dry etching a silica substrata with a reactive ion etching unit.

U.S. Pat No. 4,832,759 also discusses the generation of a plurality of surface discontinuities by means of ion beam etching. The majority of prior art studies have used photo lithographic techniques to engineer surface features with controlled morphology for the study of cell behaviour thereon. Other techniques include glancing angle deposition, laser ablation, laser deposition, replica molding of x-ray lithography masters, imprint lithography, micro contact printing and etching and ink-jet printing. For example, Canadian Pat No. 2,323,719 discusses the production of structural elevations by the LIGA lithographic process which incorporates x-ray lithography, electrodeposition and moulding. Other substrates having nanoscale topography have also been described (*Wear (1997), 41, 383-398 and Nature (1991), 352, 414-417*).

Cell-substrate interactions in the natural environment are influenced by the surface topography of the substrate, the topographical features of which are represented at the nanoscale level. Some of the above-mentioned techniques for engineering synthetic surface features are capable of generating topographical features at the nanoscale level but none has so far offered a quick and convenient means to study, manipulate or modify cellular properties at this level.

It is an object of the present invention to provide a substrate which can be used as a biomaterial for orienting cell or tissue growth.

According to the present invention there is provided the use of a substrate as a biomaterial to orient cell or tissue growth, the substrate comprising:

a base portion; and

a surface layer covering at least part of the base portion, the surface layer providing the substrate with topographical features having at least one nanoscale dimension of from about 1 to about 200 nm, said topographical features having the capacity to orient cell or tissue growth thereon and/or therebetween.

The invention provides a novel use of a substrate which may find application in a wide variety of circumstances. For example, the invention may be used to provide medical implants which orient cell growth thereon. Prior art substrates having nanoscale topography have been reported as biomaterials but there has been no satisfactory commercial development in the field of tissue or cell growth orientation. There are a wide variety of medical end uses for technology which can orient cell growth. Other examples include wound dressings, nerve regeneration materials and dental implants. "Orient" includes the exercising of directional influence over cell or tissue growth, for example influencing a cell culture to grow in a desired direction.

In the context of this document, "nanoscale" is used to refer to topographical features having at least one dimension which is measurable at the nanometre level, for example a feature which measures from about 1 to about 200nm, preferably from about 1 to about 150nm, even more preferably from about 1 to about 100nm, in at least one dimension.

The topographical features may form a random array on the surface layer of the substrate. Such an array may comprise, for example, an agglomeration of peaks and troughs, preferably having substantially the same or similar dimensions and

physical characteristics.

Alternatively, the topographical features may form an ordered array on the surface layer of the substrate. An ordered array may comprise, for example, a series of longitudinal and/or lateral ridges. A combination of random and ordered arrays may be used also. Whatever form of array adopted by the topographical features, the substrate of the invention preferably comprises nanoscale topographical features separated from other nearest neighbour similar nanoscale topographical features by distances of up to about 1000nm. For example, when the array comprises individual peaks and troughs, each peak in the array may be separated from its nearest neighbour peaks by distances of up to about 500 nm, preferably no more than about 200 nm. Where the array comprises a series of longitudinal ridges, each ridge in the array may be separated from its nearest neighbour by distances of up to about 1000 nm, preferably no more than about 500 nm.

Preferably the base portion and the surface layer are of different materials. There may be further layer(s) between the base portion and the surface layer. In other words, the surface layer may adhere directly or indirectly to the base portion.

The topographical features are preferably provided by means of controlled deposition onto the base portion of a surface layer capable of adhering to the substrate. Such adherence may be chemical or physical.

Thus, in one of its aspects this invention relates to methods for tailoring the topography of surfaces using the controlled deposition of thin films of nanoscale material onto an underlying substrate so as to modify the cellular response to the

treated surface.

Thus, in one of its aspects the invention provides a biomaterial substrate, modified with a surface of deposited nanoscale material in order to control directionally the cellular response that occurs as a result of cell contact or interaction.

In one preferred embodiment of the invention there is provided a biomaterial substrate for the study, manipulation or modification of at least one cellular or tissue behaviour or response, the biomaterial substrate comprising a base portion being provided on a surface thereof, by means of controlled deposition onto the base portion, with a substance capable of adhering to the base portion, with topographical features having at least one nanoscale dimension and a cell or tissue growth, growth inhibition or growth control region thereon and/or therebetween, the topographical features being deposited in a densely packed array with a separation between nearest neighbour similar topographical features of not more than 1000 nm.

In another preferred embodiment of the invention there is provided a method of manufacturing a biomaterial substrate for the study, manipulation or modification of at least one cellular or tissue behaviour or response comprising a base portion, depositing onto the base portion a substance capable of adhering thereto in order to provide the biomaterial substrate with topographical features having at least one nanoscale dimension the deposit being densely packed with separation between the topographical features of not more than 1000 nm, and providing the biomaterial substrate with a cell or tissue growth, growth inhibition or growth control region on and/or between the topographical features.

The base portion may comprise a single material or may comprise two or more layers of different materials. For example, the base portion may comprise a base material, such as polystyrene, covered with a layer of a polymeric or surface active agent.

The base portion may be selected from any suitable material, this depending on whether it is intended to promote, inhibit or control cell or tissue growth on the base portion material itself or only on a substance adhered thereto. Preferred base portion materials according to the invention include polymers, glasses, ceramics, carbon, metals and composites.

The surface layer is preferably formed from a polymeric material. Polyolefins and halogenated polyolefins are particularly suitable, for example poly(tetra-fluoroethylene), poly(trifluoroethylene), poly(chlorotrifluoroethylene), poly(vinylidenefluoride), poly(2,2,3,3-tetrafluorooxetane), poly(fluoroalkyl acrylate), poly(hexafluoro-propylene), poly(perfluoropropylene oxide), poly (2,2-bis(trifluoromethyl)-4,5-difluoro-1,3-dioxole), poly(vinyl perfluoroalkyl ether), poly(fluoro-alkyl methacrylate), or other polymers or perfluoroalkoxy compounds, poly(ethylene), poly)propylene), poly(isobutene), poly(isoprene), poly(4-methyl-1-pentene), poly(vinyl alkanoates) and poly(vinyl methyl ethers) as homo - or copolymers. One particularly preferred material is PTFE. Preferably the surface layer material is insoluble or sparingly soluble in water. The surface layer material is preferably able to adhere to the base portion without any additional binding material. However, additional binding agents may be used if desired and the

substrate may in that case have a multi-layer structure comprising one or more layers of a binding agent between the base portion and the surface layer.

The biomaterial substrates, methods and uses of the invention have advantages over conventional engineered surfaces used in medical engineering and methods for making them. Cell behaviour can be directionally manipulated and controlled by selecting different topographies as cells respond differently to different physical environments. The invention therefore provides a valuable tool for use in medical engineering.

This technique can be envisaged to be useful in a number of applications. One such application would be to use the substrate in the production of bioactive glass coated substrates for nerve regeneration. The technology may also prove useful in dental implants, employed to produce a gingival fibroblast attachment to the implant abutment that would secrete collagen and create circumferential tissue fibres around the implant. This would create a tight, fibrous collar, which would protect the soft-tissue attachment and reduce the incidence of peri-implantitis. These are a few applications where the technology may prove valuable, but those skilled in the art would realise that there are many other applications that may benefit from this technique.

The invention will now be more particularly described with reference to the following Figures and Examples in which:

Figure 1 shows an image of a biomaterial substrate in accordance with the invention, the image being generated by means of atomic force microscopy (AFM).

Figure 2 shows a high magnification image generated by a light microscope of cells on a biomaterial substrate in accordance with the invention.

Figure 3 shows a light microscope image of fibroblast cells on an alternative biomaterial substrate in accordance with the invention.

Figure 4 shows an image generated by a light microscope of bovine fibroblast cells on a biomaterial substrate in accordance with the invention.

Figure 5 shows a high magnification image generated by a light microscope of bovine fibroblast cells in figure 4.

Example 1

A standard glass microscope slide was washed once with methanol followed by copious rinsing with deionised water (Millipore-Q 18.2 M). The glass slide was then heated to 220 °C on a hot plate for approximately 10 minutes to allow for thermal equilibrium to take place. A thin strip of polytetrafluoroethylene (PTFE) (3 x 2 x 0.5 cm) was then applied to the glass slide once in one direction along the long axis of the slide while still on the hot plate by means of a wiping action. The glass slide was then removed from the hot plate and allowed to cool. The PTFE-wiped glass slide was then gently wetted with water to determine if the PTFE was deposited successfully as shown by the appearance of "water-lines" on the glass slide due to the hydrophobicity of the PTFE fibres. The surfaces of the PTFE coated slides were then scanned using an atomic force microscopy (AFM) as shown in Figure 1. Figure 1 shows the formation of approximately 20nm high PTFE fibres spaced apart from approximately 100nm. The next stage was to use the PTFE coated glass slide in cell

culture experiments.

A suspension of clone L 929 mouse fibroblast established cell line was prepared from a culture maintained in Eagle's Minimum Essential medium with a 5% foetal calf serum supplement. The suspension was prepared at cell concentration of approximately $1x10^5$ cell/ml. This was performed by immersing each PTFE coated slide in a cell culture medium containing established fibroblast cells for approximately 24 hours in an incubator at 37 °C.

After this period the PTFE coated slides were removed from the culture medium and examined with an inverted phase-contrast light microscope. The results clearly show that the surface influences the cell growth in the direction of the PTFE fibres, as shown in figure 2. A control (results not shown) of untreated glass slide showed no orientation or elongation of the cells, indicating that the effects observed with the PTFE/glass matrix resulted from the directionality of the PTFE fibres.

Example 2

The effect of time on the cell growth in the direction of the PTFE fibres was assessed over a 6 day period. Slides were presented with an inoculum of L 929 murine fibroblasts cells in culture medium [approx 0.2 ml cell conc. 10^6 cell/ml] and applied to the PTFE surfaces as outlined in Example 1. The dish was then incubated for 30 minutes to allow the cells to attach to the glass slide surface. After 30 mins the plate was flooded with culture medium and re-incubated. The plates were then examined by phase contrast microscope at 24 hour periods. After each time period, the dishes were removed from the incubator and the slides examined by phase

contrast microscopy. The slides were stained with methylene blue after the end of a 6 day period in order to assess areas of viable cells. The results were as follows:

After one day, a high concentration of cells was observed at the site of the inoculum. These cells were well spread and adhering to the substrate. Where the fibres were observed a small number of the cells were aligning.

After two days, the cells were clearly aligning. The cells were becoming confluent.

After six days, The cells had spread only in the direction of the applied fibres and the cells were viable.

These results suggested that cells could be grown in a directional manner by using PTFE fibres as a substrate.

Example 3

In order to assess what the effect of two PTFE fibres of differing orientations had on cell growth, PTFE coated glass slides were prepared as in Example 1, but the PTFE fibres were deposited at right angles to each other. Cell culture experiments using L929 fibroblast cells were carried out as in Example 2.

A suspension of clone L 929 mouse fibroblast established cell line was prepared from a culture maintained in Eagle's Minimum Essential medium with a 5% foetal calf serum supplement. The suspension was prepared at cell concentration of approximately $1x10^5$ cell/ml.

1 ml of the cell suspension was directly applied to both treated and untreated surface of a substrate prepared as described in Example 1. The cells were left in contact with the substrate for 30 minutes to allow cells adhesion, then the substrate

was flooded with culture medium and maintained at 37 °C/5% CO_2 for 48 hours. After this time the culture medium and non-adherent cells are removed. The substrate was then treated with 100% methanol to fix the cells and the substrate is stained with 0.04% methylene blue for 10 minutes.

Figure 3 show that the cells again aligned and elongated along the direction of the fibres but at crossover points between sets of fibres it was also observed that the cells oriented at right angles to each other. This demonstrates that by controlling the way in which the PTFE is deposited on the surface one can control the directionality and orientation of cells that are grown on that surface.

Example 4

Further experiments were directed towards the growth of bovine fibroblast cells on PTFE fibres in order to assess whether a different type of fibroblast cells would react in the same manner to the PTFE fibres as the L 929 mouse fibroblast cell line.

Primary bovine fibroblast cells were obtained from the Unit of Opthalmology, University of Liverpool at second or third passage and maintained in Dulbecco's Minimal essential Medium supplemented with 10% foetal calf serum. A cell suspension was prepared at a cell concentration of approximately $5x10^4$ cells/ml. 1 ml of this cell suspension was directly applied to the surface of a substrate prepared as described in Example 1. The cells were left in contact with the substrate for 30 minutes to allow cells adhesion, then the substrate was flooded with culture medium and maintained at 37 °C/5% CO_2 for 48 hours. After this time the culture medium and non-adherent cells are removed. The substrate was treated with 100% methanol to

fix the cells and the substrate is stained with 0.04% methylene blue for 10 minutes.

Figure 4 shows an optical micrograph detailing alignment of the bovine fibroblast cells with the PTFE fibres. The cell morphology is very different to that seen on an untreated glass base substrate or polystyrene base substrate.

Figure 5 is a higher magnification of Figure 4 showing bovine fibroblast cells in contact with nano-fibres applied to a glass substrate.

Example 5

As other cell and tissue types may have an application utilising PTFE fibres as a substrate for orientated cell growth, murine neuroblast cells were assessed to investigate their growth behaviour on the substrate.

The neuroblast cells were grown in accordance with the protocol as outlined in Example 5 and also showed evidence of alignment of cells relative to the PTFE fibres. The alignment with neuroblast cells was not as clearly defined as the alignment shown by fibroblasts, but was consistent and repeatable.

CLAIMS

1. Use of a substrate as a biomaterial to orient cell or tissue growth, the substrate comprising:

a base portion; and

a surface layer covering at least part of the base portion, the surface layer providing the substrate with topographical features having at least one nanoscale dimension of from about 1 to about 200 nm, said topographical features having the capacity to orient cell or tissue growth thereon and/or therebetween.

2. Use according to claim 1 wherein the topographical features are provided in the form of at least one regular and/or ordered array.

3. Use according to claim 2 wherein the array comprises a series of longitudinal and/or lateral ridges.

4. Use according to claim 3 wherein the ridges have substantially the same or similar dimensions and/or physical characteristics.

5. Use according to any one of claims 2 to 4 to orient cell or tissue growth in a manner at least partially determined by the configuration of the array.

6. Use according to any one of claims 1 to 5 to orient mammalian cell growth.

7. Use according to any one of claims 1 to 6 wherein the surface layer is formed from a polymeric material.

8. Use according to claim 7 wherein the polymeric material is selected from, polyolefins and halogenated polyolefins.

9. Use according to any one of claims 1to 8 wherein said topographical features have at least one dimension of less than about 50 nm.

10. Use according to any one of claims 1 to 9 wherein the surface layer comprises topographical features separated from other nearest neighbour similar topographical features by distances of up to about 1000 nm.

11. Use according to any one of claims 1 to 10 wherein the surface layer comprises topographical features separated from other nearest neighbour similar topographical features by distances of up to about 500 nm.

12. Use according to any one of claims 1 to 11 wherein said base portion and said surface layer are of different materials.

13. Use to any one of claims 1 to 12 wherein the surface layer is able to adhere directly to the base portion.

14. Use according to any one of claims 1 to 13 wherein the base portion is formed from a material selected from polymers, glasses, ceramics, carbon, metals and composites.

15. Use according to any one of claims 1 to 14 wherein the substrate is used to orient cells *in vitro*.

16. Use according to any one of claims 1 to 15, wherein the substrate is used to orientate cells *in vivo*.

17. Use according to claim 16 in a medical implant.

18. Use according to claim 16 as a wound dressing.

Figure 1

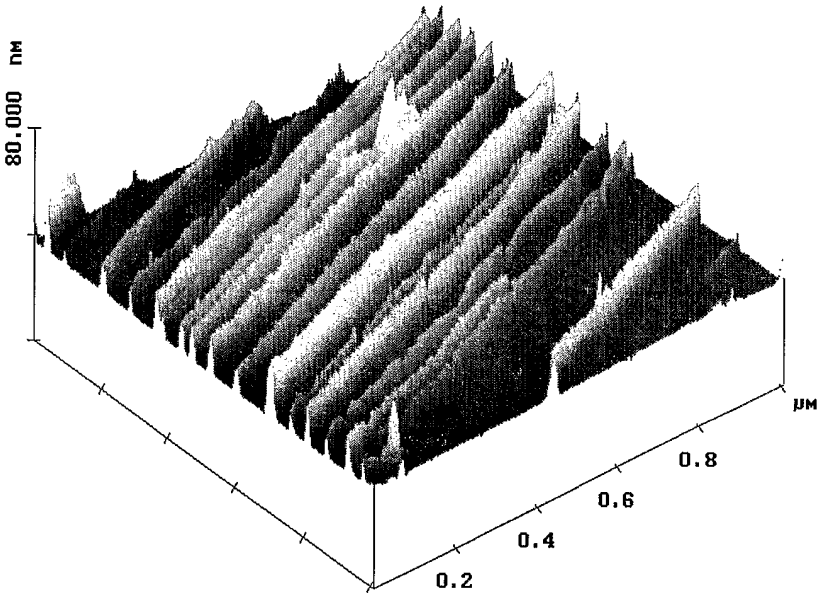

Figure 2

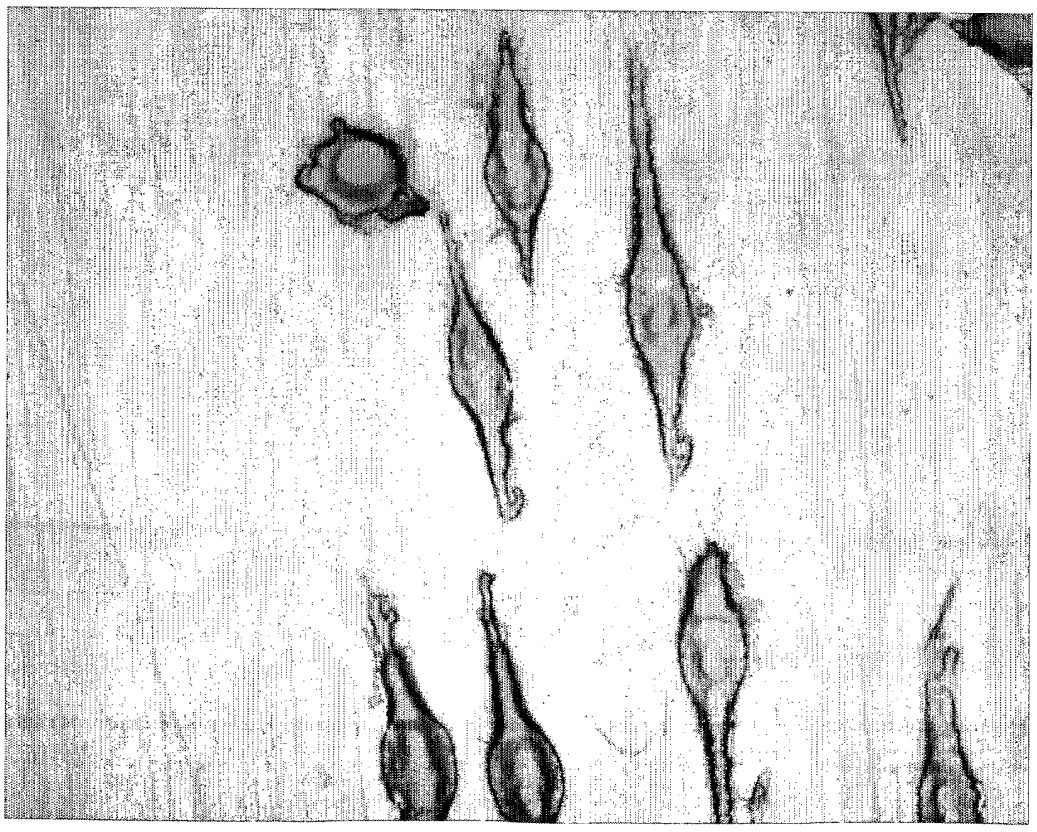

Figure 3

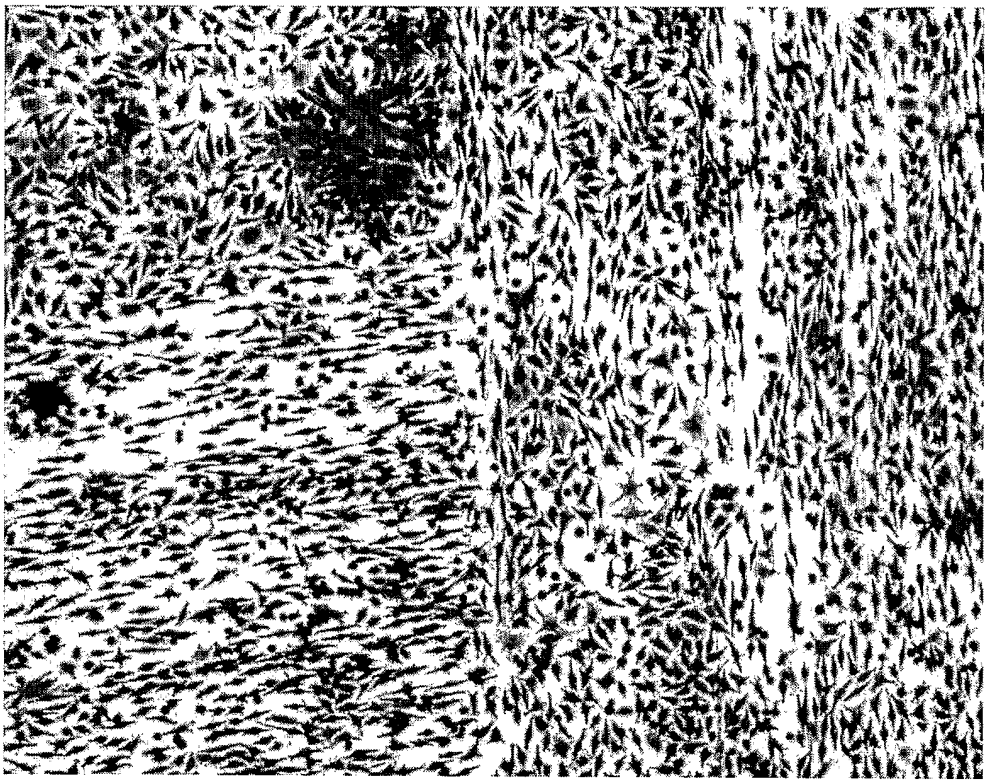

Figure 4

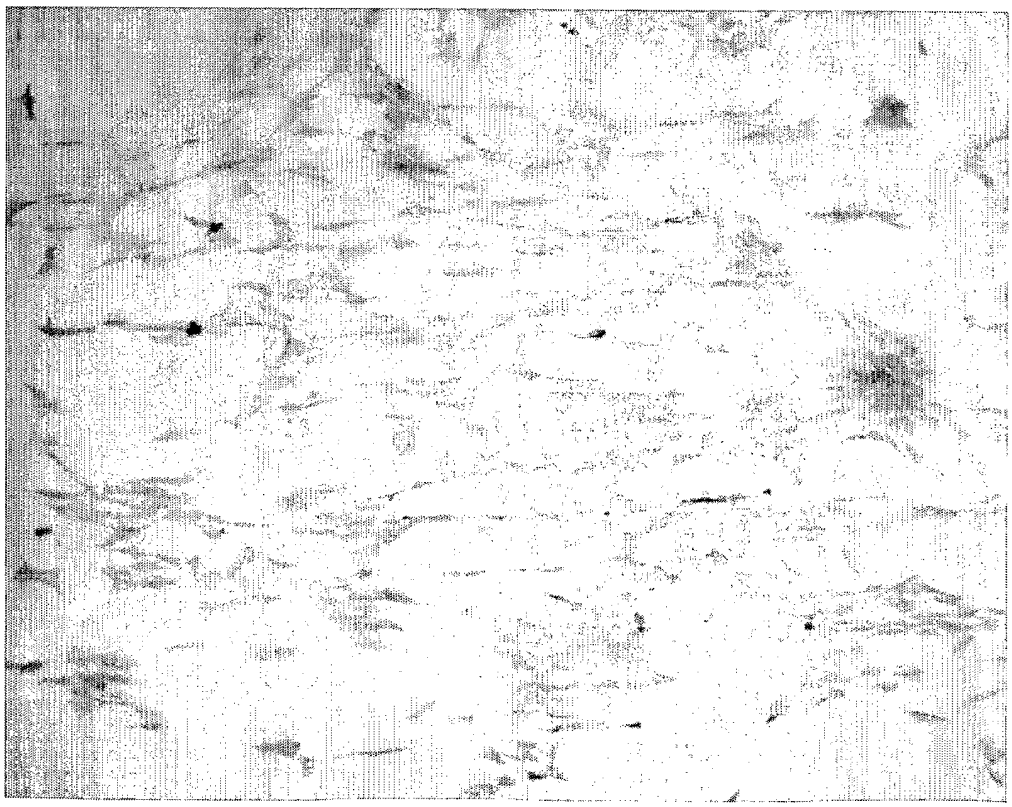

Figure 5