Nanotechniques and approaches in biotechnology

by Adam Curtis and Chris Wilkinson

Nanotechnology has enabled the development of an amazing variety of methods for fabricating nanotopography and nanopatterned chemistry in recent years. Some of these techniques are directed towards producing single component particles, as well as multi-component assembly or self-assembly. Other methods are aimed at nanofeaturing and patterning surfaces that have a specific chemistry or topography. This article concentrates mainly on surface-directed nanobiotechnologies because they are nearer to commercial realization, in applications such as tissue engineering, control of biofouling and cell culture, than those directed at producing nanoparticles.

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The discovery of the nanoworld in the past decade or so has depended to a large extent on the invention of the atomic force microscope and a variety of methods to fabricate nanostructures.

The nanoworld – natural 'nanoness'

The princess in the fairy story was so sensitive that she could feel a pea through many layers of sheets; the scale relationships are about 400 to 1. Cells seem to be even more sensitive to their environment because they can react to objects as small as 5 nm, which are some 1000–5000 times smaller than themselves. Of course cells have to get closer to the object and many adhesions of cells are made over distances of 3–15 nm from the plasmalemma lipid barrier to the surrounding objects. In vivo there is considerable detail in the surrounding environment of the cell, for instance the 66 nm banding on collagen fibres amongst which many cells live.

When cells are taken out of the body to be cultured, or when prosthetic devices, such as bone pins, are implanted, the cells encounter a very unfamiliar nanoworld in which nanodetail is chaotic, random, or even offers opposite cues to those that the cells usually receive. For example, the average plastic culture dish has ridges ~10 nm high on the culture surface (Fig. 1). The polished metal surface of the metal 'ball' on a ball and socket hip joint has a scratched surface with grooves and ridges ~20–50 nm deep. Indeed, making a truly flat surface is difficult but some crystal cleavage surfaces, such as in silicon and mica, can be flat over large areas to a few nanometres. Happily, if surprisingly, glass coverslips are also relatively flat, but polishing or similar processes do not

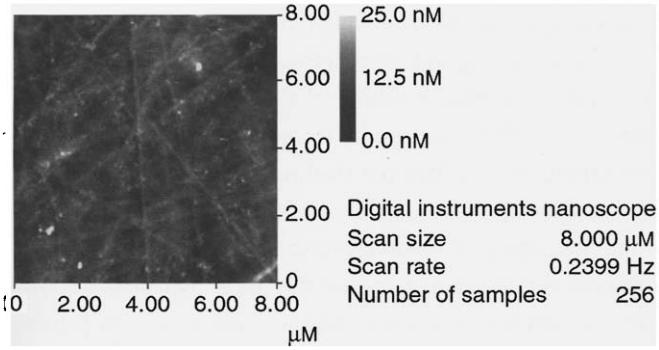

Fig. 1. The surface of a polystyrene tissue culture dish viewed with the aid of a Nanoscope IIIa atomic force microscope used in the contact mode. Dark areas are depressed, white areas raised. Vertical scale range (black to white) 30 nm.

yield good quality surfaces. The surface chemistry, as well as the topography, can also be chaotic on the molecular scale. For these reasons alone we need to know more about the reactions of cells to the nanoworld and how to control them.

Small is odd – an unexpected world

The macroscopic world can be scaled down through orders of magnitude to the microscopic scale with little or no change in expected properties. Knowing the mechanical properties of a 1 mm cube of glass allows the prediction of the mechanical behavior of a large sheet of plate glass or even of a glass mountain (if there were one). This is not the case as you enter the nanoworld; the gate to which is ~100–300 nm in dimension. Interfacial forces begin to become of great importance, quantization effects emerge and everything has to be re-thought. Indeed, our experiences in this world have made us realize that our conventional knowledge, derived appreciably from colloid chemistry, is not adequate for understanding nanostructures. These experiences suggest that nanostructures will provide a very effective experimental tool for studying the physical chemistry of complex systems such as colloids. Gleiche *et al.*[1] and Fradin *et al.*[2] have produced examples of different types of interface effects on the nanoscale. Interestingly, several attempts have been made to define the dimensional limits of objects that should interest the student of the nanoworld and several have opted for the 100 nm limit, but perhaps it should be larger at the 300 nm gateway.

Do cells react?

Despite the obvious opportunities for cells to have an interesting nanometric environment in which to live, do they respond to such features? The evidence is becoming increasingly clear that they do for many varieties of animal cell but the evidence for bacteria is less clear. The answer to this question could not be sought until reliable ways of reproducing relatively large areas of nanopatterned surfaces were developed. By the late 1980s it was already known that many vertebrate cell types responded to micrometric topography, but effects for some cell types were larger for deep structures in the 5–10 µm depth ranges than they were for shallower structures. There was, therefore, an expectation that the effects might disappear at approximately the 1 µm level.

Clark[3] was sceptical about this limit and, using holographic photolithography, produced a surface with grooves 130 nm wide and 9 µm deep. Epithelial cells cultured on this surface responded by alignment of isolated cells and of the cytoskeleton to the grooves even when grown in sheets of adherent cells, although the cell outlines were not aligned. This aroused interest in the possibility that nano-features might affect cells, but at that time nanofabrication was poorly developed and so progress was slow. However, Wojciak-Stothard[4] found a simple way to produce features of

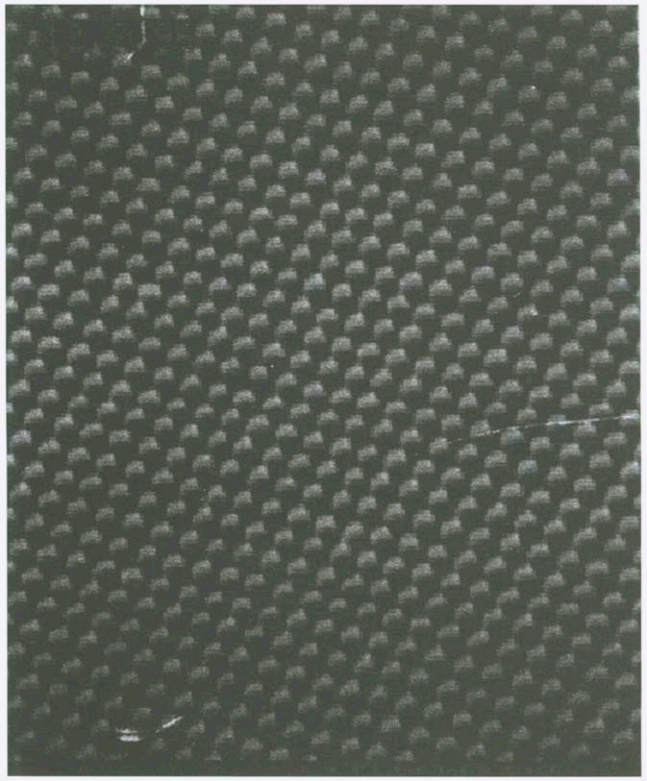

Fig. 2. Scanning electron micrograph of silica pillars. Fabricated in fused quartz using electron-beam lithography. Center-to-center spacing is 50 nm. (Courtesy of B. Casey, Glasgow University, UK.)

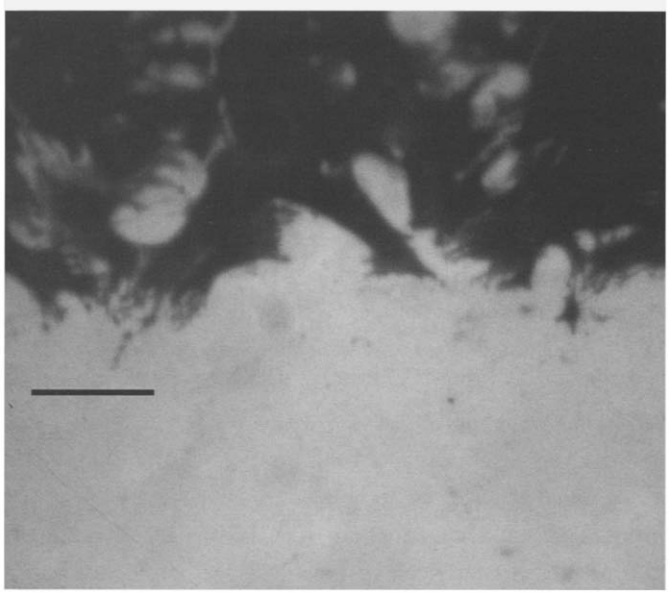

Fig. 3. Low adhesion on a nanopatterned surface. Low-power light micrograph of rat tendon cells (dark stained), grown on a polycaprolactone surface cast from solvent solution of a 300 nm center-to-center silica structure. The clear area free from cells is the surface structured with nanopits, shown in detail in Fig. 6. The culture period was 21 days. No cells are found on the nanostructured area. The scale-bar represents 10 microns. (Courtesy of J. Gallagher, Glasgow University, UK.)

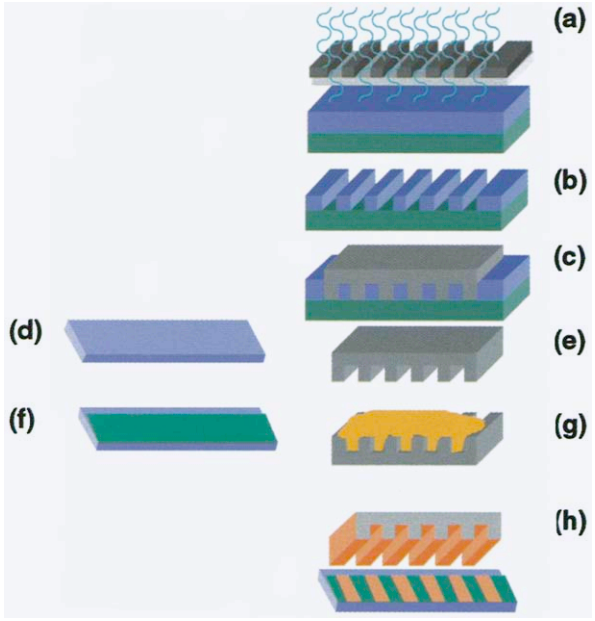

Fig. 4. Microcontact printing. The method of printing strips of protein. (a) A thick (>5 mm) photolithographic resist is illuminated through a mask with the desired design. (b) The illuminated areas are specifically dissolved. (c) A polysiloxane polymer (Sylgard 187 Dow Corning) is cast onto the resist and cured at 90°C. (d) The surface to be patterned is cleaned and prepared (i.e. glass with 3-aminopropyl, 3-aminoethyl triethoxysilane). (e) The Sylgard stamp (made with or without a backing) is removed and cleaned. (f) The surface is chemically activated by using a crosslinking agent (i.e. glutaraldehyde). (g) The stamp is inked with a solution of the desired peptide or after thorough cleaning, the stamp can be reused several times. (Courtesy of Dr M. Riehle, Glasgow University, UK.)

nanometric depth by reducing the dry etch time. This method produced grooves that were micrometrically wide, millimetrically long and nanometrically deep. Cells of fibroblastic, endothelial, epithelial and macrophage types responded to these grooves by increased adhesion and orientation. At that time the shallowest grooves were 44 nm deep. Later, Rajnicek[5] used some of these structures and found that nerve cells responded to 5 nm steps. These cell types reacted to the nanometric structures by changes in cell adhesion, alignment and orientation of the cells, cytoskeletal orientation and changes in cell activation[3,4,6–9].

As a result of collaborations between cell biologists and electronic engineers, we have begun to produce nanofeatured surfaces in silica. Regular arrays, such as that shown in Fig. 2, reduced the adhesion of several cell types even though the flat unfeatured surface outside the 'etched' area allowed good adhesion of the same cells (Fig. 3). To replicate these structures cheaply and quickly, a reversed master in silica was produced so that casts, embossing or injection moulded polymers, would have the desired structure. Thus, if large areas of nano-pits are needed the master is made with nanopillars or projections. Interstage secondary masters can be produced using a variety of methods so that the final product is identical to the original master. The techniques used are identical to those involved in the production of long runs of a CD and the accuracy of replication can lie at the 2 nm level. Although this is not the only way of making masters, it is the preferred way of copying a structure.

The interesting feature of the silica dots is that they were probably the first structure that was made for biological use on the nanoscale in *x*, *y* and *z* dimensions. Previous structures had usually been on the nanoscale in only one or two of the three dimensions.

Alternative nanofabrication methods

The main methods of nanofabrication used currently are listed in Table 1, but it is important to note that the pace of development is so fast that new methods are emerging on a monthly basis. Colloidal resists are colloidal materials, such as gold metal sols or other very small particulate materials, which if spread on a surface will form a random or semi-random distribution of particles that can then be used as an etch resist and later removed (or left if desired). The pattern of the particles is transferred into the substratum by the etch.

Microcontact printing[10,11] is so simple a concept that it is familiar to children - and most of us are amazed that it works so well. A photolitho-graphically patterned stamp, usually made from polydimethylsiloxane, is fabricated with the desired pattern etched to a very shallow depth in the surface. This stamp is then loaded from a protein, polysaccharide or other large molecule-bearing surface carrying these molecules in a weakly attached or unattached form. If hydrophilic proteins or peptides are to be printed then the surface of the stamp needs to be made hydrophilic, for example by a very short-term etch with oxygen plasma. The stamp is then brought into contact with the surface that will carry the print. That surface already bears a cross-linking reagent, such as glutaraldehyde, attached, for example, to aminopropyl triethoxy silane. The stamp is left in contact for about an hour and then removed. The protein is then found to be transferred to the surface, for example an appropriate polymer bearing the aminopropyl triethoxy silane (Figs. 4 and 5).

Monolayer assembly of thiol compounds on a gold substratum[12] provides an interesting research process but is unlikely to have commercial potential because it does not allow transfer to an embossing, casting or moulding system.

Recently, a range of new technologies has appeared. These include 'rapid prototyping'[13], self-assembling processes[14] and new developments of diaelectrophoretic patterning[15].

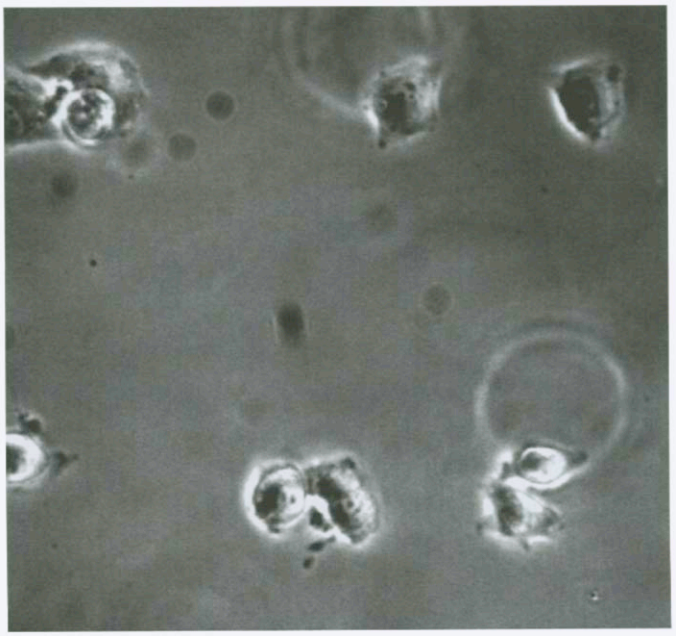

Fig. 5. Phase contrast view of mouse endothelial cells grown for 16 hours on a glass coverslip, microcontact printed with alternate stripes of albumin (no adhesion) and beta-thymosin sulfoxide. The cells adhere to the beta-thymosin sulfoxide. The cells line up 50 mm apart, corresponding to the width of the albumin stripes.

Characterizing the surfaces

Unfortunately, the majority of methods for characterizing surfaces are ones that average over fairly large sampling areas. However, scanning electron microscopy (SEM), atomic force microscopy (AFM) and other scanning probe microscopies (SPM) give good images of surface topography at high resolution (Fig. 6). The latter two microscopies can operate in many different modes, such as force modes that allow force pattern or surface mechanical properties to be measured. AFM has the potential for direct chemical mapping of the surface, but this has not been taken very far as yet.

Table 1. Current methods of nanofabrication

Type	Materials	Resolution
Electron beam lithography[a]	Silica, silicon Silicon nitride Silicon carbide	*x*, *y* and *z* to 10 nm
Colloidal resists[a]	On any of above	*x*, *y* and *z* to 5 nm
Self-organizing (assembling) systems	Polymer demixing Self-assembling particles and monolayers Other self-assembling systems	In 10 nm ranges
Microcontact printing	Any fairly large molecule	*x* and *y* to 200 nm *z* to one monolayer
Embossing, casting or injection moulding From master made by above methods	Most polymers Some metals	Down to 21 nm
Particle synthesis	Many materials (e.g. superparamagnetic beads)	Upwards from 3 nm diameter

[a] Followed by dry or wet etch.

Chemistry or topography?

One area of controversy is whether the cells react to local patterns of chemical difference or to features such as topography. Obviously, microcontact printing is intended to produce patterns of chemistry and the self-assembling systems should do the same. But the direct writing of a pattern into an isotropic substratum, such as fused quartz (silica), should in theory produce a surface that lacks patterned chemical features and the replication of such a surface by embossing into polymer should have a similar result. It is possible that slight defects or anisotropies in the etch process or embossing process produce small very localized chemical differences, but this is at present a slightly sterile argument because we do not yet possess the chemical means to make surface analyses with such high resolution.

One argument favoring the existence of topographic reactions of cells runs as follows. Cells react in similar ways to the same topography on surfaces that are chemically very different. For example, silica dots (such as those in Fig. 2) have much the same effect on cell adhesion as the same surface made in polycaprolactone or polyurethane. It can of course be argued that the cells are reacting to a layer of protein adsorbed onto the surface and that this accounts for the similarities, but this leaves the question of why the topography should produce differences in adsorption.

Britland *et al.*[16] competed topography against chemistry to see which produces the greatest alignment of neurites. The extending neurites of nerve cells were offered a choice of aligning to chemically printed strips of the protein laminin or to grooves crossing the laminin strips at 90°. The shallowest grooves lost out to the chemical tracks in this competition, but when the grooves were deeper than 500 nm topographic effects overwhelmed the chemical ones.

Box 1

What features of nanopattern do cells react to?

- Imbalanced or asymmetric distribution of interfacial forces across the nanostructure induce concentrations of attractive or repulsive forces at particular places, for instance electrostatic forces of repulsion might be concentrated at sharp projections.
- Strains within the cells induced by their attempt to conform to the non-planar surface.
- Stress relief in the substratum surface - induced partly by random thermal events and partly by the cells themselves - is likely to exert mechanical forces on cells.
- Patterns of surface chemistry possibly displaying binding sites for specific molecules.

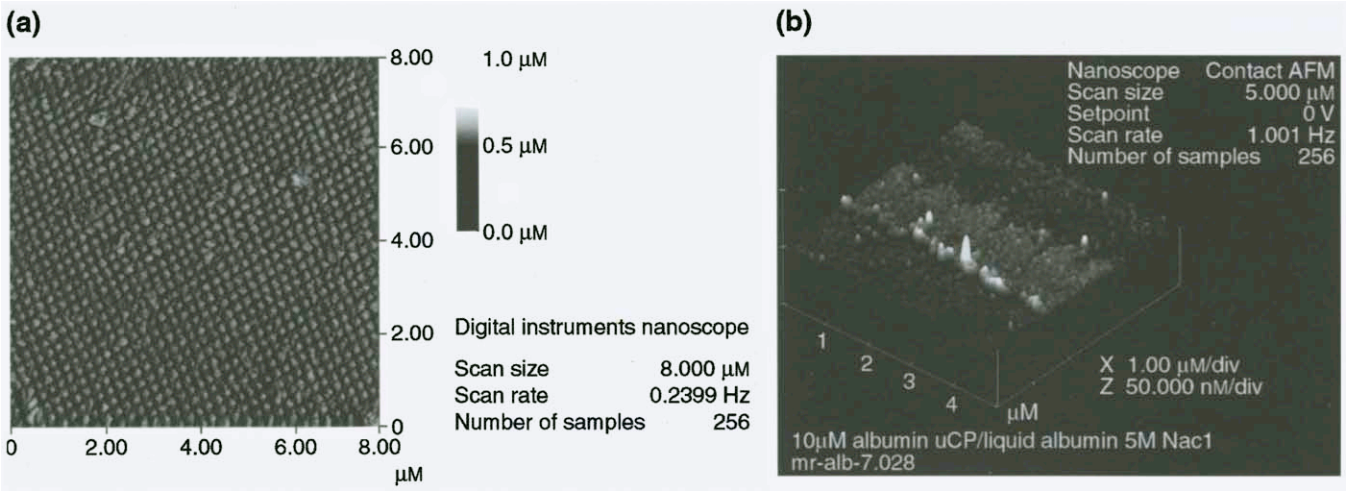

Fig. 6. The figure shows atomic force microscopic views of nanostructures. (a) Polycaprolactone pits (as shown in low power in Fig. 3) after 21 days culture. (Nanoscope IIIa contact mode image courtesy of J. Gallagher.) (b) Microcontact printed albumin strip viewed using AFM. Note the large difference between horizontal and vertical magnifications when interpreting this image. The 'globular' structures are appreciably larger than single albumin molecules.

Box 2

Biotechnological uses of nanotopography: main areas

Examples of devices for controlling cells where nanofeatures form part of the device

- Cell traps (prisons and death cells)
- Cell orienters and movement stimulators
- Collagen aligners
- Biosensors (not yet made at the nanoscale)
- Nanoanalysers (e.g. for DNA and specific proteins)

Examples of complete devices

- Nanosamplers (e.g. of cell surface; to be fully realized)
- Nanoimplanters (e.g. of artificial surfaces, DNA, drugs)
- Magnetic nanoparticles for drug delivery, cell destruction
- Cell moving and assembling devices
- Low adhesion/high adhesion surfaces for biomedical uses (e.g. stents)
- Nanomachines

Chemical tracks appear to give cell alignment cues, but the premise of the argument can be reversed and the statement made that tracks of chemicals do have a finite thickness so that the cells might be reacting to the topography of the track. It is noteworthy that cells aligning on tracks are often aligned to the edge of the track.

What do cells react to?

If the cells are reacting to physical forces in and induced by the substratum rather than to specific chemical bonding, then a large range of possibilities should be considered (Box 1).

Products and possible products

At the moment this area is one of promise and perception and not of complete achievement. Starting by considering effects of nanotopography, any system where you wish to have very low adhesion or very high adhesion, especially in an embossable non-degradable or bio-degradable polymer, is potentially useful in a wide variety of biotechnological devices or biomedical uses. The systems depending on surface topography or surface immobilization of chemicals are likely to be relatively durable because the features are strongly attached to the surface and relatively cheap to produce because printing or embossing processes can be used. There will probably be two types of products: (1) those whose primary purpose is to provide high or low adhesion, for example in devices to prevent tissue adhesion; and (2) those products whose primary purpose is different but where low (or high) adhesion is required to improve performance of the device, for instance to prevent fouling of biosensors.

Many other types of nanodevice for biomedical and biological use can and have also been envisaged. Adhesion might be important in these devices, but nano-detection of single or very small numbers of molecules, nanometric movement and charge flow devices will also be important. Products that are being realized or are generally accepted as goals that should be achieved can be seen in Box 2.

Ingeniously baroque?

Two years ago, a review of a meeting on 'nanotechnology in biotechnology'[17] described several speakers likening nanobiological devices to those of Rube Goldberg as 'inelegant but ingeniously baroque'. I find this a curious statement. Cells live in a nano- or micro-featured environment. Natural selection would act to remove redundant reactions and redundancy is the spirit of the baroque. Devices we make are likely to operate correctly only if they interact with cells in much the same general way as the environment does; so the route into this world ought to be one in which we systematically examine the reactions of cells to simple well-defined structures.

Moreover, this area of scientific endeavour is a truly interdisciplinary one and any effective progress, even on a single project, needs contributions from physicists, engineers, material chemists, biophysicists, cell and molecular biologists, as well as end users such as orthopaedic surgeons.

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Glossary of terms

Biofouling - Biological fouling (biofouling) is the undesirable accumulation of microorganisms, plants and animals on artificial surfaces.

Cytoskeleton - Part of the cytoplasm (contents of a cell) that remains when organelles (subcellular particles of membrane-bound organised living substances) and internal membrane systems are removed.

Endothelial cells - Type of cell that lines the cavities of the heart, blood and lymph vessels.

Epithelial cells - Type of cell that covers the internal and external surfaces of the body, including the lining of vessels and other small cavities.

Fibroblastic - Appearance of fibroblasts (though may not actually be fibroblasts) - i.e. connective tissue cells.

Lipid - Any of a heterogeneous group of fats and fatlike substances characterised by being water insoluble and extractable by nonpolar solvents such as alcohol, ether, chloroform, benzene, etc. All contain aliphatic hydrocarbons. Lipids act as a fuel source, are a costituent of cell structure and serve other biological funtions. Lipids include fatty acids, neutral fats, waxes and steroids.

Macrophage - Type of cell from mamalian tissue that plays a role in killing of bacteria, protozoa and tumour cells and releases substances that stimulate the immune system.

Neurites - In tissue culture, hairlike projections of neurons stimulated by growth factors and other molecules. Neurite may refer to any filamentous or pointed outgrowth of an embryonal or tissue-culture neural cell.

Plasmalemma - The plasma membrane of a cell.