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Rapid Production of Transparent Micro/Nanostructured Polymer Substrates for Biomedical Surface Interaction Studies

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ABSTRACT

The increase in the number of investigations into biomedical interactions with structured substrates has produced a necessity for transparent, micro- and nanostructured surfaces. To facilitate research, it must be possible to use these structured surfaces in conjunction with established culturing and imaging apparatus available within a biomedical laboratory. Such surfaces can be produced using a rapid fabrication technique where freestanding, transparent, structured polymeric substrates are produced for use in cell-surface interaction experiments. The production method is based on a nanoimprint lithography (NIL) technique using silicon based moulds to emboss structure into the surface of biologically compatible thermoplastic polymers. The moulds are fabricated via deep reactive ion or focused ion beam lithographies. The polymers can then be sized to fit in existing cell culture plates, or can be employed with reusable silicon-based culture plates.

Unstructured polymers fabricated using this technique have transparencies rivalling that of glass, and although the transparency of the micro- and nanostructured films is reduced slightly, they are still sufficiently transparent to be used with conventional transmission microscopes. Cells cultured on the micro/nanostructures can therefore be viewed both from above and through the substrate, allowing cell-structure interactions, which would normally be hidden by the bulk of the cell, to be viewed. The optical transparency of the structured polymers also allows them to be used in

fluorescent techniques. The possibility of chemically functionalising the polymer surfaces further increases the useful applications of these substrates.

KEYWORDS: Nanostructure, microstructure, polymer, embossing, surface functionalisation, biomedical applications.

CONTENTS

1. INTRODUCTION

It is natural for scientists and engineers to mimic nature in trying to develop applications relevant to both biological and medical fields. This is especially true for applications utilising structured and patterned surfaces. Although methods for the structuring of surfaces, at dimensions comparable to those commonly found in nature (i.e. at size ranges from micrometres downwards), has been available for some time, it is only lately that the technology has been available to synthetically fabricate such morphological and chemical surface patterns with controllable geometries.

It has long been known that the morphology of surfaces plays an important role in biology. Biological systems that may be affected range from the production of 3-dimensional (3-D) tissues, to the alignment of individual cells on 2-dimensional (2-D) structures. The effects of topological structuring do not, however, simply end at the positioning and orientation of cells. Various studies are now discovering that the interaction of a cell with a structured surface can also affect the adhesion properties of the cell to the surface [1, 2, 3] and even the function of stem cells [4].

As with many techniques, the need to produce physical structures at very small dimensions can be traced back to the electronics industry. Moore's Law [5] describes the need for an increasing number of transistors on a single integrated circuit. This need has consequently led to improved lithography methods for transistor production. Fortunately, the appearance of these new lithographic techniques has had a knock-on effect in opening other application areas which benefit all branches of the physical sciences and society in general; for example, as the basis for micro-electro-mechanical systems (MEMS), microfluidics, and microarray technology.

The production of micro- and nanoscale chemical patterns has been another challenge which, through careful development, has lead to a number of breakthroughs. Surface patterning techniques, such as microcontact printing [6] and dip pen lithography [7], can be used to produce chemical patterns with dimensions down to sub-micrometer levels over large areas [8]. Such techniques can also be used for the deposition and alignment of more than one chemical species. In areas such as microarray patterning, this has lead to the development of on-chip sequencing and diagnostic systems [9, 10].

Improvements in lithography and chemical patterning, along with a greater understanding of material and surface properties, have allowed experts within the biomedical field to collaborate in producing surfaces useful for a range of applications. One example of a group of increasingly useful materials is biodegradable polymers.

These can be used in polymeric surgical implants (such as screws) which are degraded by the body over time, and hence do not require a second operation for removal [11]. Knowledge of the surface properties of the polymer has helped to ensure that the implants heal the affected tissue without being rejected by the host body.

Here we present a method for the rapid production of micro/nanostructured polymeric surfaces for in-vitro biomedical cell-surface interactions. We describe examples of micro and nanostructuring methods which can produce a regular topological structure at micron and sub-micron dimensions, and explain why polymer surfaces formed in this way have advantages over techniques using inorganic materials, especially when it comes to fabrication, diversity of chemical and physical properties and biocompatibility. We then describe methods of surface chemical functionalisation, concentrating on polymer surface modification, which allows tuning of the surface chemical and physical properties to be undertaken. Finally, we present some results showing how micro/nanostructured polymeric surfaces can be prepared, cheaply and quickly, using compression moulding techniques, and how they can be used for in-vitro cell-surface interactions in biomedical applications.

2. SURFACE MICRO/NANOSTRUCTURING TECHNIQUES

2.1 Topological surface micro/nanostructuring

There are a number of methods for the structuring of surfaces on micron and sub-micron levels. Traditionally, before the advent of more advanced fabrication technologies, these relied on mechanical or chemical treatments of the surface, that imparted structuring, but in a manner that was not well controlled. For example, mechanical polishing or chemical etching of metals and inorganic materials (e.g. glass and silicon) can produce surfaces with regular structuring at micron scales, but at smaller dimensions the patterning is much more random [12].

2.1.1 Mask-based lithography micro/nanofabrication techniques

The advent of mask-based photolithography has increased the control of surface patterning at micron scales. These days, the best photolighographic/chemical etching methods can achieve sub-micron resolutions [13]. This advance in particular has led to the establishment of microengineering disciplines for microelectromechanical systems (MEMS) and microfluidics, miniaturised total analytical systems (μ-TAS) and miniaturised laboratory devices (lab-on-a-chip) [14].

Other developments, such as the invention of the electron beam lithography (EBL) technique, and the later development of deep ultraviolet (UV) and x-ray lithography techniques, have further expanded the field of mask-based lithography. However, the latter techniques suffer from problems with source and mask production, respectively [15]. Therefore, EBL is the method of choice for structure production using polymer resists (such as poly(methyl methacrylate) (PMMA)) with a resolution down to 10 nm [15].

Post-lithographic pattern transfer processes have also been improved. Reactive ion etching (RIE), has allowed the fabrication of structures with aspect ratios superior to those developed using wet etching techniques, with increasingly vertical side-walls and much smoother surfaces [16].

However, one problem that dogs mask-based lithography techniques is the need to use a mask and the use of potentially harmful photoresist chemicals that must be disposed of carefully. The mask must have a lateral feature resolution suitable for the technique, a non-trivial task when sub-100 nm features are required. Wet etching compounds the problem of chemical safety/disposal, as it uses other, inherently corrosive, chemicals for the etching of the inorganic substrate. Further, a combination of photolithography and etching requires two steps, which increases the possibility of errors and is time consuming (although there have been recent developments in mask-less lithography [17, 18]).

2.1.2 Direct-write and other nanofabrication techniques

Lately, direct-write techniques, such as focussed ion beam (FIB) milling [19] or laser milling [20] have been developed. In both techniques material is directly removed from the surface of the substrate. The techniques use focused, computer-controlled, high-energy beams: ions for FIB and light for laser milling respectively. In such methods, the need for a mask and a variety of chemicals is negated. However, at this time, the resolution of these direct-write techniques is limited by the spot-size of the beam that can be focussed on the substrate surface. In laser milling the spot size is limited to the micron range [20], whereas for FIB milling it can be as low as 30 nm [21].

Other direct-write nanofabrication techniques suitable for the production of surface structures include scanning probe techniques, epitaxy and molecular self-assembly [15]. The scanning probe techniques (e.g. nanomanipulation, dip pen etc.)

have the advantage of being highly controllable, but the disadvantage that they are serial and difficult to scale up for large-scale production. Epitaxial methods are limited to inorganic substrate modification, but do have the advantage that they can be used to pattern large areas.

Similarly, self-assembly techniques can be used to pattern large areas, but they are hampered by the presence of impurities and, sometimes, the need to use complex chemical production methods. However, a problem for the examination of biological specimen stems from a lack of methods for the preparation of well defined organic surfaces. Surface assembled monolayers (SAMs), particularly those formed by the adsorption of long-chain alkanethiols, are well suited for studying interactions of surfaces with proteins and cells. The control the composition and properties of the SAM, combined with the simple methods that can pattern their functional groups in the plane of the monolayer, makes this class of surfaces eminently for fundamental studies of protein adsorption and cell adhesion.

2.2 Polymer substrates

Mask-based and direct-write micro/nanofabrication techniques allow structuring of the surface of inorganic substrates at micron and sub-micron scales. However, many inorganic and metallic materials are not biocompatible, and indeed, with the exception of glass, none of the aforementioned inorganic substrates, which are eminently suitable for micro and nanostructuring, are regularly used in the biological laboratory for in-vitro cell culturing applications.

However, biologists regularly use organic materials in the form of polymer-based Petri-dishes and multi-well culture plates. Although such apparatus may not primarily be intended for surface culture studies (normally they contain a culture medium or a gel suspension), it is inevitable that cells will come into contact with the polymer surface at some time. Consequently, the polymer must not affect the culturing of the cells in any adverse way. Fortunately, a number of polymers are biocompatible, and a subset are even biodegradable over short time scales (i.e. months), a characteristic which can be used to reduce waste and allow for the production of implants that can, for example, be absorbed by the body [11].

2.2.1 Embossing micro/nanofabrication techniques

Due to their diverse characteristics, polymers also have a large number of possible fabrication methods. These include vacuum forming for the production of food containers, blow moulding for drinks bottles and dip moulding for plastic safety gloves. Lately however, one technique has been developed for the production of micro and nanostructures on thermoplastic polymer surfaces. In bulk manufacturing industries, the technique is called compression moulding. In academic circles, it is more commonly called hot embossing or nanoembossing, techniques used as the basis for hot embossing lithography (HEL) or nanoimprint lithography (NIL) respectively [22]. Typically, this technique uses a mould to impart structure into a polymer surface (Figure 1). The polymer is heated to above its glass transition temperature (T_g), causing it to soften. The mould can then be forced into the polymer, under pressure, causing the softened polymer to fill the recesses in the mould surface. Cooling the system to below T_g, then causes the polymer to harden within the mould and take the shape of the surface structures in the mould surface. The pressure can finally be released and the two pieces can be separated. This leaves the polymer replica with a structured surface which is a negative copy of that on the mould.

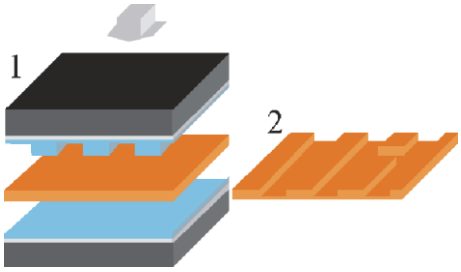


Figure 1. Schematic diagram of the embossing technique used to produce micro/nanostructures in a polymer surface. The polymer is placed in a press, heated to above its T_g, and embossed under pressure using a stamp with a micro/nanostructured surface (1). The entirety is then cooled to below T_g before the stamp is removed, leaving the structured polymer surface (2).

HEL and NIL have the advantage that they are parallel techniques, which allows a number of replicas to be produced simultaneously. They are also relatively fast fabrication methods and can be used with a wide variety of polymers. The techniques can be adapted for use with low temperature [23] and photocurable polymers [24].

2.2.2 2-D versus 3-D polymer surfaces

Some publications that have examined surfaces for culture studies have questioned the use of 2-D structured surfaces when dealing with biological media [25, 26]. They suggest that 3-D structured surfaces offer a more accurate matrix for examining cell-surface interactions. Indeed, a number of research groups are concentrating on the production of 3-D polymer matrixes, typically for use as scaffolds for tissue regeneration. In most cases, these biomedical scaffolds are fabricated using a single polymer, such as poly(lactic acid) (PLA), which is structured to produce an inhomogeneous latticework of holes within the polymer matrix [27]. This is commonly achieved using fibrous or foam-like polymers that can spontaneously produce a lattice structure [28], and can even be biodegradable [29]. Hydrogels are similarly used as space filling scaffolds, scaffolds for bioactive molecule delivery, and scaffolds for cell delivery [30].

In almost all these cases, the matrix is fabricated by producing porous polymers, either from scratch or by using bulk polymer, and creating the porosity through some other means. Unfortunately, both fabrication techniques mean that control of the pore size in the matrix is difficult and it is possible that a distribution of pore sizes is achieved. Step-wise techniques which aim to rectify this problem include fused deposition modelling [31] and 3-D NIL [32] where layers of polymer structures are built up, one on top the other, to produce a 3-D lattice. These methods produce a much more ordered 3-D matrix and allow more control over the pore size.

2.3 Topological micro/nanostructure characterisation

Structures at micrometer scales are commonly characterised by optical microscopy, interferometry or mechanical profilometry techniques. In the latter case, a mechanical stylus is simply drawn across the surface of the substrate to be examined with a force low enough not to damage the surface. The horizontal and vertical displacement of the stylus is then measured to give a sectional profile of the surface. By raster scanning the stylus across the substrate surface a more detailed 3-D picture can be produced. The vertical and horizontal resolutions of a stylus profilometer depend on the dimensions of the stylus used, the scan speed of the stylus and sampling rate of the apparatus, but vertical resolution at the tens of nanometers range can be achieved. Typical instruments can scan areas of up to a few centimetres; a property useful when trying to image a large area.

For optical measurement methods, white light is used to build up an image of the structures being examined. Optical microscopy merely uses reflected, or transmitted, light to produce a direct 2-D image of the surface. White light interferometry, on the other hand, uses an interference pattern, produced by redirecting some of the incident light onto a mirror set at a distance relative to the focal length of the lens, to image the surface. The light reflected from the surface of the substrate and the mirror is then directed back towards a CCD detector. When the substrate is in focus, the light patterns interfere with each other and this interference is detected by the CCD detector. The lens can then be incrementally moved through the z-direction, allowing it to focus on various surfaces on the substrate at different heights. By recording the height at which the lens is focussed and recording the interference pattern, the interferometer can build up a 3-D image of the surface, with nanometer vertical resolution, in a very short space of time. Unfortunately, the lateral resolutions of both these optical techniques are limited by the wavelength of the light used (~200 nm).

Unfortunately, as previously described, the dimensions of some of the structures that can be formed on polymer surfaces are below 200 nm. For these structures, characterisation is usually performed using scanning electron microscopy (SEM) or atomic force microscopy (AFM). In SEM, a beam of electrons is focussed on the substrate surface and secondary electrons emitted by the surface molecules are detected. Again, by scanning the surface of the substrate, a 2-D image of the surface features can be attained. With an experienced operator, a SEM image can be produced very quickly with nanometer horizontal and vertical resolution. Unfortunately, the method suffers from the disadvantage of electrostatic charging of the surface of non-conducting materials. This can be overcome, to some extent, by coating the surface with a thin metallic layer, however, with a subsequent reduction in resolution.

AFM is a technique that is similar to that of the surface profilometer, but at a much smaller scale and with a much higher versatility; although with a consequent increase in system complexity [33, 34]. A stylus is moved over the surface of the sample, as in the profilometer. However, in this case, deflection of the stylus is measured by reflecting a laser beam off the back of the cantilever and measuring beam displacement using a quadrant photodetector. The AFM can be used in a number of different measurement modes depending on the characterisation required (e.g. topological, electrical, friction etc.) and on the surface to be examined (e.g. hard, soft, rough etc.) [34]. The AFM technique is increasingly being used in biology [35] and, as

with the SEM, an experienced user can produce an AFM image at nanometer resolutions relatively quickly. One disadvantage of this technique is that positioning during imaging is limited to the maximum displacement of the piezoelectric elements used to position the substrate, therefore measurements on large area samples may require unloading and reloading of the substrate.

2.4 Summary

A number of fabrication techniques are available for topological micro- and nanostructuring of inorganic and organic substrate surfaces. The advent of techniques for small-scale structuring of polymers, via compression techniques such as HEL and NIL, over large areas and in a parallel fashion, makes polymers ideal for the production of micro- and nanostructured surfaces for biomedical applications. These can be based on a number of polymer types, including those already in use in biological laboratories, depending on the required properties. The development of 3-D fabrication techniques, in their infancy at the present time, will provide even more tools for cell-surface interactions.

3. SURFACE CHEMICAL FUNCTIONALISATION TECHNIQUES

3.1 Chemical functionalisation

Although a number of different materials are available for the production of structured surfaces, problems of bio-incompatibility, due to surface properties such as a high hydrophobicity, limit on the number of useful surfaces for such interaction studies. Therefore, there is a need to be able to alter the surface properties of substrates, which are biocompatible or otherwise, to increase the number of surfaces available for study: this is normally achieved via chemical functionalisation techniques. Suitable functionalisation molecules can be designed which have a “linking” moiety at one end, which attaches it to the substrate surface, and a functional moiety at the other end, which produces a functionalised surface for the cells to interact with, either directly or via further chemical modification. Typically, these techniques are used either to change the surface properties of the surface, chemically or physically, to make them more attractive to biological species, or to produce a surface for direct interaction with biological species through the attachment of a biochemical moiety to the substrate surface.

3.1.1 Direct functionalisation

Normally, direct functionalisation is achieved using molecules based on silanes or thiols, which are chemically linked to the substrate surface, via a silicon bond or a sulphur bond respectively, and which spontaneously form SAMs across the substrate surface under carefully controlled reaction conditions. Further, the functionalisation molecule is usually based on a long-chain alkyl group that helps the SAM to form and holds the functional group away from the substrate surface. The fact that such molecules can form SAMs means that the production of such a functionalised surface can be relatively easy. One example is the functionalisation of silicon-based surfaces using trichloro(tridecafluoro-octyl)silane (Figure 2). The silane is simply deposited in the vapour phase within a dessicator. The silane attaches to the silicon surface, **1**, with the loss of a chlorine atom, **2**. Subsequent molecules align with the attached molecules, **3**, and the SAM is finally prepared via a baking step at 80-90 °C which causes cross-linking between the silane molecules **4**. Excess silane molecules are then washed off using hexane.

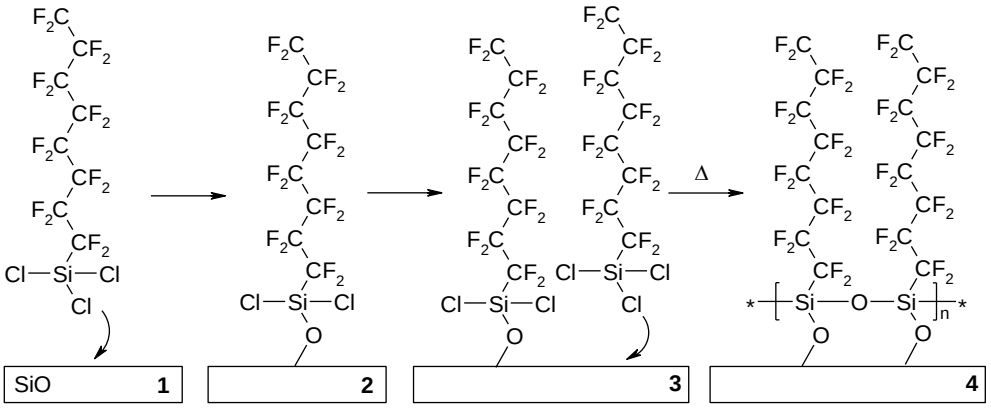


Figure 2. Functionalisation of a silicon surface using trichloro(tridecafluoro-octyl)silane to alter the surface properties; to increase the surface wettability for example (see figure 13).

3.1.2 Indirect functionalisation

The functionalisation of a surface can also be changed through chemical alteration of the end groups of SAMs previously deposited on the surface. As an example, figure 3 shows a chemical pathway for the alteration of an ester-terminated alkylsilane, **1**, attached to a suitable substrate surface. After the initial grafting of the silane to the substrate, the ester termination moiety can be converted to acid, **2**, by immersing the functionalised substrate in hydrochloric acid for 12 hours. This can

subsequently be changed to a succinimide terminated alkylsilane, **3**, by reaction with N-hydroxysuccinimide (NHS) in the presence of N, N-dicyclohexylcarbodiimide, and finally to an amide terminated surface, **4**, by reaction with ammonia in ethanol solution. Each step produces a different alkylsilane termination moiety, which gives the surface different chemical and physical properties.

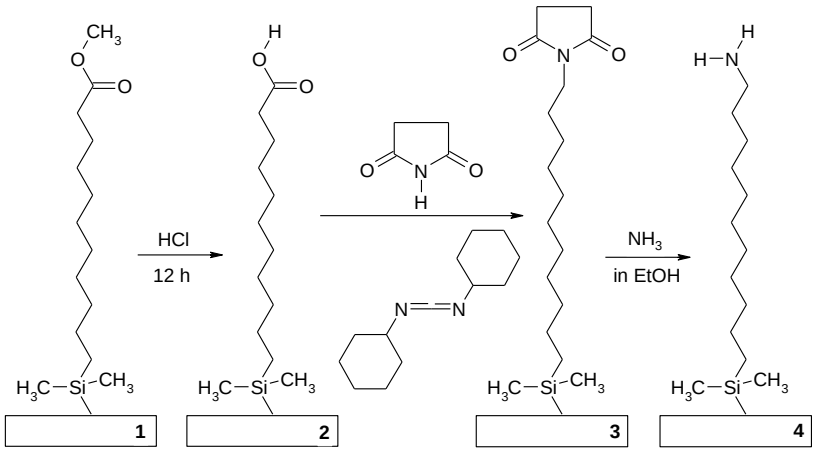


Figure 3. Reaction scheme outlining the chemical transformation of an alkylsiline giving different functional surfaces. These chemical functionalities alter the surface properties of the functionalised substrate surface, for example via a change in hydrophobicity (see figure 4).

3.1.3 Surface wettability – an example of one effect of indirect chemical modification

As an example of the change in physical properties, functionalisation can be used to drastically alter the surface wettability of a substrate. Via careful choice of functionalisation groups with different termination moieties, the hydrophobicity of the surface can be "tuned". For example, the contact angle of a water droplet on the surface of a silicon oxide surface can be altered between 30° and 130°, from hydrophilic to hydrophobic respectively, by the addition of various silane-based functionalised molecules (Figure 4).

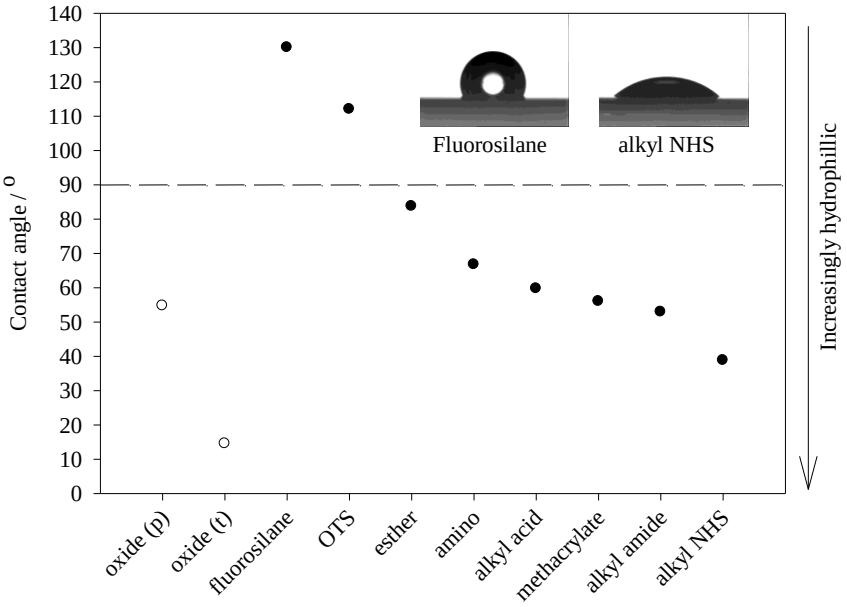


Figure 4. Advancing contact angle measurements of silicon oxide surfaces prior to chemical modification (○) and when functionalised (●). The dotted line indicates the transition from hydrophobic to hydrophilic. Inset are optical images of the water droplet on the fluorosilane and alkyl NHS functionalised surfaces. Key: **oxide (p)** = pristine silicon oxide surface; **oxide (t)** = piranha treated silicon oxide; **fluorosilane** = trichloro(tridecafluoro-octyl)silane; **OTS** = (octadecyl)trichlorosilane; **ester** = (10-carbomethoxy)decyl-dimethylsiloxane; **amino** = 3-(2-aminoethylamino)propyl-trimethoxysilane; **alkyl acid** = acid-terminated decyl- dimethylsiloxane; **methacrylate** = 3-(trimethoxysilyl)propyl methacrylate; **alkyl amide** = amide-terminated decyl- dimethylsiloxane; **alkyl NHS** = N-hydroxysuccinimide terminated decyl-dimethylsiloxane.

3.2 Polymer surface functionalisation

Several techniques have been used to tailor the properties of polymer surfaces and to introduce functional groups, such as amine or carboxylic acid moieties, that can be used for the covalent coupling of bioactive molecules. Available functionalisation techniques can be classified in three groups, namely plasma treatment, chemical modification and grafting.

3.2.1 Gas discharge techniques

Gas discharge techniques have been extensively used for the functionalisation of polymer surfaces [36, 37, 38]. The surface modification is obtained by exposing the surface to a partially ionized gas, which has the advantage of modifying the polymer surface to a depth of only 50 nm [39]. Plasma treatment processes have also been used for the introduction of functional groups onto polymer surfaces: the new group being introduced by changing the gas used in the plasma treatment. Typically, the plasma

treatment can change the surface chemistry and the wettability of the polymer surface, and has the advantage that it is quick and does not require complex chemistry. Table 1 lists some examples of functionalisation of polymer surfaces using different plasmas.

Table 1. Polymer surface functionalisation using different plasmas.

Plasma gas	**Polymer**	**Added surface functional groups**	**Ref.**
O_2, Ar	Polypropylene (PP), Polystyrene (PS)	C-O, C=O	40, 41
O_2	Poly(dimethylsiloxane) (PDMS)	C-O, C-O-C, C=O	42, 43
NH_3	Polyethylene (PE)	C-NH_2	44, 45
NH_3	Poly(paraphenylene terephthalamide) (Kevlar®)	C-NH_2	46
NH_3	Poly(ethylene terephthalate) (PET)	C-NH_2	47
N_2O/Ar	Poly(ethylene terephthalate) (PET)	C=O, aldehyde	48

3.2.2 Chemical modification of polymer surfaces

To chemically modify polymers, a wide range of chemical surface reactions can be used in liquid or vapour media. Figure 5 gives examples of how polymer surfaces can be modified. Chromic acid, or oxidants such as permanganate or periodate, can be used to introduce carboxylic acid moieties on the surface of PE [49]. For PS it is possible use paraformaldehyde, in acidic solution, to introduce OH groups that can be further derivatised to carboxylic acid using bromoacetic acid [50]. In some special cases, functional groups present within the polymer structure can be used. In the case of PMMA and PET, basic media produces the hydrolysis of surface ester groups to yield acid groups [50, 51]. PMMA can also be hydrolised in presence of a diamine to produce amine terminated surfaces [52, 53]. Similarly, in the case of Kevlar®, a polyaramide polymer, hydrolysis can be used to produce surface amine groups [54].

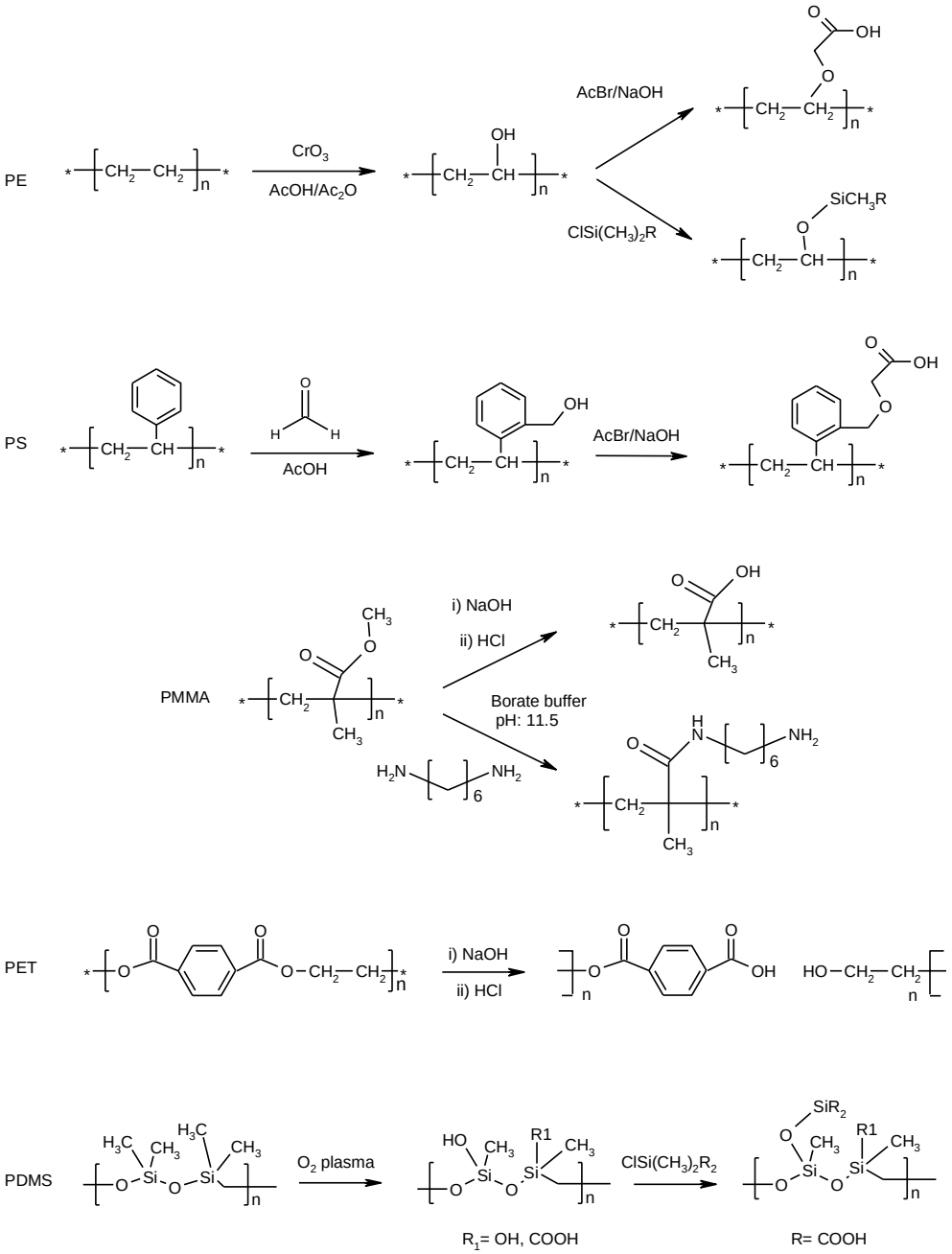


Figure 5. Examples of the surface modification of PE, PS, PMMA, PET and PDMS by chemical means to produce functionalised polymer surfaces with active chemical moieties.

A combination of the previous methods (i.e. plasma treatment and chemical functionalisation) allows further modification of the surface of the polymer, e.g. for the addition of a silicon-based layer to the polymer surface. PDMS and PE can initially be treated with O_2 plasma to introduce OH groups on the surface. Post plasma treatment exposure of the surface to an alkyltrichlorosilane vapour causes the silane to form a monolayer film on the polymer surface. This introduction of OH groups on the polymer surfaces and subsequent treatment with the silane is similar to the reactions commonly

used to treat silicon oxide surfaces. Similarly, $SiCl_4$ can be used to produce a homogenus layer of silicon oxide over plasma oxidized PE [55]. The surface of this layer has a low roughness, is densely and uniformly functionalised, and provides functional groups that can be used in the formation of alkylsiloxanes SAMs on the polymer surface.

3.2.3 Grafting

Several methods exist for the grafting of chemical functionality to a polymer surface. Typically, free radicals or peroxides are formed at the polymer surface by exposing it to alpha radiation, electrons, UV radiation, plasma or ozone, or via chemical treatment [56, 57]. The energetic active sites that are formed are then used as surface bound initiators which initiate grafting reactions with unsaturated compounds that contain the desired functionality (Figure 6). For example, UV radiation has been used to activate a PDMS surface to introduce by graft polymerisation acrylic acid, acryl amide, dimethyl acrylamide, and 2-hydroxyethyl-acrylate [58]. Unfortunately, grafting processes suffer from the production of polymer residues which can be formed by the unsaturated compounds, and which may cause problems post-functionalisation unless they are removed.

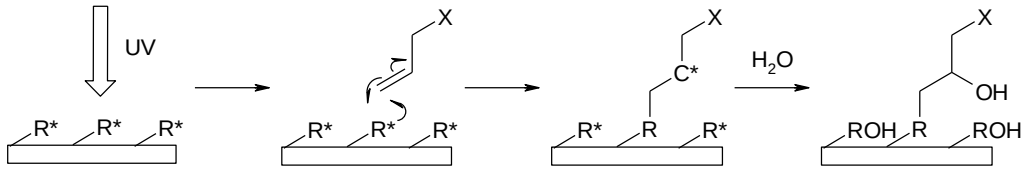


Figure 6. An example mechanism of the grafting of a molecule with a functionalised moiety (X) to a polymer surface. R* is an excited moiety on the polymer surface.

3.3 Functionalised layer characterisation

In addition to the techniques mentioned in section 2.3, several methods are available to *physically* characterise surfaces that have been subjected to surface modification, i.e. with respect to layer thickness and surface coverage. Optical techniques used to characterise thin films on flat substrates include ellipsometry [59], scanning near-field optical microscopy (SNOM) [60] and surface plasmon resonance (SPR) [61]. The ellipsometer reflects light of known polarization off a sample surface, and measures the polarization change upon reflection. The exact nature of the polarization change is determined by the sample's properties (e.g. thickness and

refractive index). Although optical techniques are inherently diffraction limited, ellipsometry exploits phase information and the polarization state of the light, and can achieve Angstrom resolution. The technique is applicable to films with thicknesses from less than a nanometre to a micrometer, and is used to study the formation of monolayers on substrates and how the layer thickness of absorbed protein layers depends on the surface chemical properties [62].

Near-field optical imaging involves illuminating a surface through a sub-wavelength sized aperture. The surface is positioned within the near-field regime of the source, in an area where the radiation from the source does not have the opportunity to diffract before it interacts with the sample. The resolution of the system is therefore determined by the aperture diameter, as opposed to the wavelength of light used. For example, if white light is used, sub-200 nm resolution can now be achieved, a resolution unobtainable using far field optical microscopy [63].

The SPR uses the evanescent field created by a beam of light shining on the surface of a thin metallic layer to measure the thickness of thin layers deposited on the metal surface. Light energy, from the incident light, interacts with the delocalised electrons in the metal film (plasmon) thus reducing the reflected light intensity. As a functional layer is added to the immobilised surface, the local refractive index changes, leading to a change in the angle of the surface plasmon resonance. This can be monitored by detecting changes in the intensity of the reflected light. The rates of change of the SPR signal can be analysed to yield apparent rate constants for the association and dissociation phases of the reaction. Thus the thickness of the added film can be calculated. As the SPR signal depends only on binding to the immobilised template, it is also possible to study binding events from biomolecules from heterogeneous solutions [64].

Other techniques that can be used to study the physical properties of functionalisated surfaces include x-ray photoelectron spectroscopy (XPS), and time-of-flight secondary ion mass spectrometry (TOF-SIMS), both of which also provide information on the chemical composition of the surfaces.

XPS is a non-destructive, surface sensitive technique that provides quantitative, surface chemical state information for all elements except hydrogen and helium [65]. The sample is irradiated with a beam of monochromatic soft X-rays causing photoelectron emission to occur from the atoms in the sample. The distribution of kinetic energies from the sample is then directly measured by the electron spectrometer.

Atomic orbitals from atoms of the same element in different chemical environments are found to possess different binding energies. Differences in oxidation state, molecular environment and co-ordination number all provide different chemical shifts. Photoelectron binding energy shifts are, therefore, the principal source of chemical information. It should be noted that these shifts can be very small and can only be detected using a high performance instrument with suitable software. Layer thickness can be measured to a depth of 10 nm.

TOF-SIMS uses a pulsed primary ion beam to desorb and ionise species from a sample surface [66, 67]. The resulting secondary ions are accelerated into a mass spectrometer, where they are mass analyzed by measuring their time-of-flight from the sample surface to the detector. An image can be generated by rastering a finely focused beam across the sample surface. Due to the parallel detection nature of TOF-SIMS, the entire mass spectrum is acquired from every pixel in the image. The mass spectrum and the secondary ion images are then used to determine the composition and distribution of sample surface constituents. Depth information is produced by removing successive layers of material from the surface which can be used to build up a 3-D map of the material under study. Unfortunately, this does mean that the technique is destructive.

Other techniques can be used to produce further information about the chemical and physical nature of the surface [68]. Some examples are given in Table 2.

Table 2. Examples of some techniques for the characterisation of surfaces

Technique	**Surface characteristic**
Various spectroscopies (e.g. FTIR, Raman, UV/visible)	Chemistry
X-ray photoelectron spectroscopy	Chemical structure/electrical properties
Transmission electron microscopy	Atomic structure
Contact angle measurement	Wettability/Surface energy

3.4 Protein immobilisation - an example of an application for chemically functionalised surfaces

Modelling of the adsorption of proteins to surfaces with different functional groups approximately correlates with the surface hydrophobicity. The protein can be reversibly attached to the surface via hydrophobic or electrostatic interactions. These

interactions are the most common and experimentally the simplest, but they are difficult to control and normally have no positional directionality. The adsorption on hydrophobic surfaces is often kinetically irreversible. However, the protein layer can be removed using detergents or replaced by other proteins with a higher affinity for the surface [69]. This is advantageous when proteins are to be substituted, but disadvantageous when the protein layer is to remain in place.

Methods that rely on covalent coupling of proteins to surfaces are inherently more controllable and give layers of protein that cannot dissociate from the surface or exchange with other proteins in solution. The most successful techniques for attachment of proteins using covalent immobilisation have been based on the formation of amide and disulfide bonds [70]. Unfortunately, one problem that can occur when the protein is immobilised is protein denaturation, which consequently causes a loss in protein activity. A remedy for this problem has involved coupling of the protein to "inert" materials, such as oligo(ethy1ene glycol) terminated SAMs.

The biospecific adsorption of proteins to SAMs is important for biological applications, such as chromatography, cell culturing, the production of artificial implants and organs etc. Typically, the target protein must be allowed to interact with the surface, to the exclusion of all other proteins. A common immobilisation technique is based on the biotin-streptavidin interaction [71, 72]. The effectively irreversible complex formed in this system is useful for many applications, including the immobilisation of proteins. The strepavidin allows the introduction of anti-biotin sites on the surface [73], which can be used to introduce a wide range of biotinylated biomolecules, such as the reversible binding of lactate dehydrogenase to SAMs that present analogues of nicotinamide adenine dinucleotide (NAD) [74, 75], and the recognition of immobilised antigen by antibodies [76].

Contrary to surfaces for the immobilisation of protein, materials that resist the adsorption of proteins have also been investigated [69]. An efficient technique for producing a protein resistant surface has been to use poly(ethy1ene glycol) (PEG) layers, which can be produced by adsorption, covalent immobilisation, or radiation cross-linking. Polymers with carbohydrate moieties in their conformation can also be used, but the addition of PEG is ultimately the most efficient polymer-based technique. Another available method is to use a "blocking" protein, such as bovine serum albumin (BSA). Unfortunately, such proteins tend to denature over time, or exchange with other proteins in solution, allowing unwanted protein access to the surface.

3.5 Summary

Polymers present a wide range of surface functionalisation possibilities. These can be used to alter the surface properties of the polymer itself, or by the implementation of an additional functional layer, or can be used to add chemical or biological functionalisation. The possibility of patterning such chemical functionalisation and utilising more than one functional species, using such techniques as micro-contact printing or dip pen lithography, opens up a number applications within array and sensor production applications.

4. BIOMEDICAL APPLICATIONS

Both micro- and nanostructured surfaces have been found to affect the growth of cells cultured on the surface. Inorganic materials that have been used for topographically structured surface interactions with cells include elements, such as gold and silicon, and compounds, mainly based on silicon: organic surfaces are normally based on polymers [77]. Important characteristics that must be defined when attempting to examine cell-surface interactions depend on the topographical properties, including structure depth, groove width etc., and the chemical/physical properties of the surface, such as hydrophobicity etc. The latter substrate characteristics are doubly important if the surface has been functionalised. The interaction between the cell and the surface can then be characterised by examining properties such as whether the cell can be made to grow on the structured surface, whether the cell propagates, how the cell orientates itself with respect the surface structures, and whether any physical or chemical changes occur in the cell, e.g. elongation [1, 78]. For tissue forming applications, these considerations are joined by the obvious need for the cells to form tissue structures with neighbouring cells, e.g. for tendon repair [79]. The applications for such structures can be grouped into a number of categories, namely those for imaging, those for adhesion, proliferation and differentiation, those for cellular structuring, and those for tissue formation.

4.1 Imaging

Transparent substrates are a preferred choice for use in a biomaterials research laboratory, a precursor to general use in the field of biomedicine. Current techniques used in biology become awkward when they have to be applied to biomaterials applications. To evaluate the biocompatibility of materials, a quantitative evaluation of

cell adhesion and proliferation generally implies the detachment of cells from a surface. However, it is very important to follow the culturing of the cells over time without interfering with or destroying the cells. The use of transparent polymers allows the researcher to study cell behaviour by means of an inverted optical microscope, the most common technique available in cell biology labs, and record the changes in cell morphology during adhesion, mobility and proliferation. Such transparent polymers can be preferred to glass as it is possible to functionalise and structure the surface to trigger specific cell responses.

4.2 Adhesion, proliferation and differentiation

Cell adhesion to surfaces in the biomedical domain implies two distinct phases. Firstly, the attachment of the cell to the surface, which occurs rapidly and involves physicochemical linkages between cells and materials by means of ionic forces, van der walls forces, etc. Second is the adhesion of the cell to the surface which it has become attached. This occurs over a longer time span and involves biological molecules, such as extracellular matrix (ECM) proteins, cell membrane proteins and cytoskeleton proteins, which interact together to induce signal transduction, promoting the action of transcription factors and consequently regulating gene expression [80].

Good interactions between cells and the surfaces of materials are highly relevant and contribute to the clinical success of implants. Surface characteristics, such as topography, chemistry and surface energy, determine whether the adhesion between cell and surface is good, and hence whether subsequent proliferation and ECM production occur to form new tissue. Individually, these characteristics establish how biological molecules will absorb to the surface and in what orientation. Thus, they determine cell behaviour and proper tissue formation.

The organization of surface roughness is an important parameter. In-vitro, several authors have determined the effect of grooves and ridges to orientate epithelial cells and fibroblasts. Orientation has also been observed in osteoblasts. Osteoblasts cultured on Ti surfaces with microrough features exhibit reduced proliferation and enhanced differentiation when compared to cells grown on tissue culture plastic or smooth Ti substrates [81]. In vivo, surface roughness may control tissue healing and enhance implant success [82, 83] However, in vivo studies have failed to demonstrate differences in bone-to-implant contact between comparable roughness parameters performed using different techniques. On the other hand, recent studies using dental

implants with a good control of the surface roughness at the microscale exhibit increased pull-out strength. The microtopography of the implant in the study included a mixed morphology of craters and micropits [84, 85].

4.3 Cellular structuring

Micropatterned surfaces can be used to understand how cells respond to specific structural features. Micrometer and/or nanometer scale topographies affect different aspects of cell behaviour, including such characteristics as cell adhesion, cell proliferation, cell differentiation, cell morphology, cell orientation, contact guidance, tissue organization, etc. [77, 80, 86, 87, 88]. Depth and width of grooves can determine how cells in the osteoblast lineage form focal contacts and how they produce and mineralize the ECM [89, 90]. However, the response of osteoblasts to microstructures cannot be considered without also considering the surface chemistry. Cell response cannot be predicted from studies performed on different materials albeit with the same surface architecture.

The production of surfaces with a well defined microarchitecture will facilitate the study of cell behaviour when it is essential to design materials capable of modulating cell response. For example, a study has demonstrated that cell attachment depends on cavity spacing, cell growth depends on cavity dimensions, and cell morphology depends on the presence of sub-micron-scale structural features [81]. Another study, performed on titanium surfaces with increasing surface roughness, concluded that osteoblasts do not react to 10 μm diameter cavities, simply sitting on top of them. However, cells were seen to enter 30 μm and 100 μm cavities and adopt different morphologies: adopting a 3-D shape within the 30 μm cavities but a more flattened one within the 100 μm cavities [91].

With regards nanostructured topographies, different cell types have been shown to react differently to different nanostructures [77]. One consequence of this is that the cell morphology on the submicrometer scale favours the formation of long and numerous filopodium [91]. However, there is probably a limit to the feature size to which the cell is sensitive. For example, Scotchfold et al. presented a study on chemically patterned surfaces and observed that osteoblasts did not react to nanometric surfaces (20 nm) on Ti/Ti surfaces [92]. Even so, it is obvious that the behaviour of

cells is greatly influenced by the micro- and nanotopography, singly or in combination, of surfaces.

4.4 Tissue formation

Tissue engineering is a growing field within biomedical research. The optimisation of the surface topography and chemistry of materials is designed to induce a faster adhesion of cells and a more efficient production of ECM, for healing wounds for example. The biomaterials used to aid tissue regeneration have to have several characteristics, including biocompatibility, and tissue conducting and tissue inducing properties. The use of polymers, in the case of bone engineering for example, is an interesting option. The use of biodegradable materials especially offers several advantages over the metals and other non-degradable materials used at present as orthopaedic implants. Firstly, they do not have to be removed after implantation, negating the requirement for a second surgical procedure and avoiding possible complications. Secondly, such polymers are easy to load with growth factors which can be used to increase cell growth and bone formation. Finally, they are liable to surface modifications to control their surface properties and consequently direct cellular adhesion and cellular response [80, 93]. Together with the possibility of fabricating composite materials, with organic molecules such as collagen or cells, these materials are a potential solution for bone grafting requirements.

Amongst other applications, besides bone grafting and regeneration, biodegradable polymer stents are currently being researched for use in cardiology [94] and urinary applications [95].

5. RAPID REPLICATION TECHNIQUE

Briefly, our technique for the production of structured polymer surfaces consists of a number of well defined steps. Topological structuring is achieved using polymer embossing. An inorganic mould is produced and used to emboss the polymer at elevated temperature and pressure to impart topography. Chemical patterning is achieved using micro-contact printing. A similar mould to that used for embossing is used to produce an elastomeric stamp. This is inked with the required functionalising molecules and then used to transfer these molecules to the polymer surface in areas where the stamp and polymer make conformal contact. In each case, the patterned polymer surfaces are used

to examine their interaction with cells. The details of each of the production steps are outlined in the following sections.

5.1. Silicon mould fabrication

Silicon moulds were produced using a previously described method [96]. For the microstructured surfaces, designs utilised included several types of surface structures, with dimensions ranging from 1 μm^2 to 100 μm^2 (i.e. 10x10 μm^2). The microstructures were defined in the mould surface to give moulds with positive superficial structures (where the features are higher than the surface) or negative superficial structures (where the features are lower than the surface). In total, the repeated microstructures cover an area of 1 cm^2.

Production of moulds in silicon oxide (SiO_2) and silicon nitride (Si_3N_4) was completed using two different fabrication methods depending on the height of the required structures. The first method involved the growth of layers of oxide and nitride on the surface of a silicon wafer previously patterned using deep reactive ion etching (DRIE). This allowed relatively tall (~1 μm) structures to be produced. In the case of the Si_3N_4 moulds, an underlying SiO_2 layer is necessary to compensate for the intrinsic stress in the thermally evaporated layers.

The second method for fabrication of the SiO_2 and Si_3N_4 moulds involved thermally growing oxide or nitride layers on a pristine silicon wafer, prior to photolithographic patterning and DRIE. This technique limited the height of the structures to the thickness of the deposited layers. In addition to these fabrication conditions, each mould was produced in two further configurations; one using the original 500 nm thick silicon wafer, modified as described above, and one where the 500 nm thick silicon wafer is anodically bonded to a 1 mm thick pyrex wafer, prior to modification, to increase the strength, and hence the lifetime, of the mould.

Further mould production was possible by modification of the unstructured silicon-based substrates, or the previously described microstructured moulds, with the introduction of micro- or nanostructures using FIB milling. The FIB (Strata DB235; FEI Co., Netherlands) apparatus could be used to mill structures into the raised structures on the original mould, or to create new patterns on the unstructured substrates. Figure 7 gives an example of a microstructured master that has been modified using FIB.

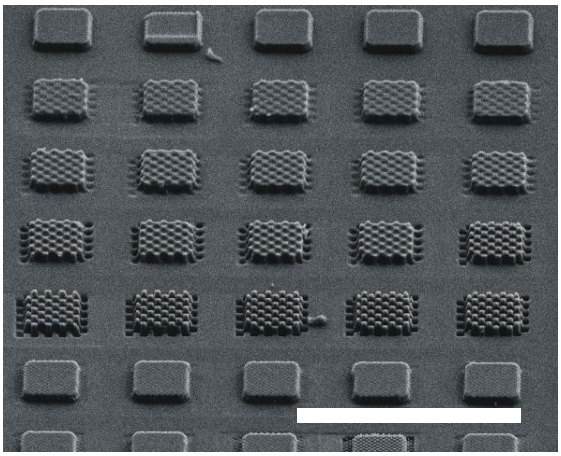


Figure 7. SEM image of a silicon substrate containing 400 nm tall, 5x5 μm^2 microstructures, some of which have been modified using FIB milling [bar = 20 μm].

The SiO_2/Si_3N_4 layers on the mould surface were used to prevent adherence problems between the mould and the polymer; however, after surface modification using the FIB a fluoroalkylsilane monolayer (trichloro(tridecafluoro-octyl)silane; United Chemical Technologies, USA), was sometimes added to eliminate any sticking problems. The fluoroalkysilane was deposited from the liquid phase using a previously described method [97]. Once prepared, the moulds could be used either for micro/nanoembossing or for the preparation of stamps for micro-contact printing.

5.2. Polymer embossing

Polymer embossing was achieved via nanoembossing using a commercially available nanoimprinter [2.5' Nanoimprinter, Obducat AB, Sweden.]. Normally the polymer used for the embossing is spun down onto the surface of a suitable substrate; commonly a piece of the material used to produce the mould. The mould is then placed in contact with the polymer surface and the embossing proceeds in a typical fashion [98]. This has the disadvantage that the embossed substrate is no longer transparent, and hence less useful for biomedical applications. To remedy this, we have developed a method for producing freestanding, embossed polymer films with high transparency [99, 100].

The polymer embossing has been achieved in 125 μm thick sheets of PMMA (Goodfellow Ltd., UK). For each experiment, the polymer was cut to the approximate size of the mould to be used for the embossing. The polymer was rinsed with isopropanol (IPA, Aldrich Chemical Co., UK), to remove any dust particles, and dried

using a stream of nitrogen gas.

A schematic diagram of the nanoembossing process is given in figure 8. The silicon mould is placed onto the base of the nanoimprinter with the structured surface to be embossed uppermost. The polymer sheet is then placed on top of the mould, with the surface to be embossed in contact with the mould. They are then both covered with a second sheet of polymer (e.g. Teflon®) with a glass transition temperature (T_g) higher than that of the polymer to be embossed. The Teflon® is used to eliminate any patterning of the backside of the polymer by the thin aluminium sheet used to hold everything in place in the apparatus (Figure 9). A drawback to this technique is that the back side of the PMMA is slightly wavy, possibly suggesting an uneven applied pressure. This does not however affect the surface being embossed and could be rectified by using a thicker piece of Teflon®, which should distribute the pressure more evenly.

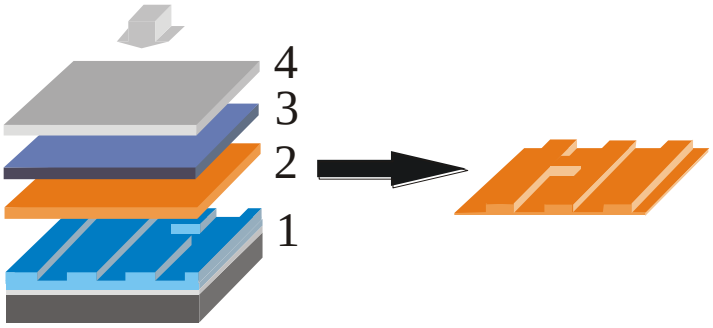


Figure 8. Schematic diagram of the embossing process used to produce freestanding polymer films containing micro/nanostructures. The PMMA (2) is sandwiched between the mould (1) and the aluminium (4) used in the nanoimprinter. The PMMA is protected by a high T_g polymer (3, see text).

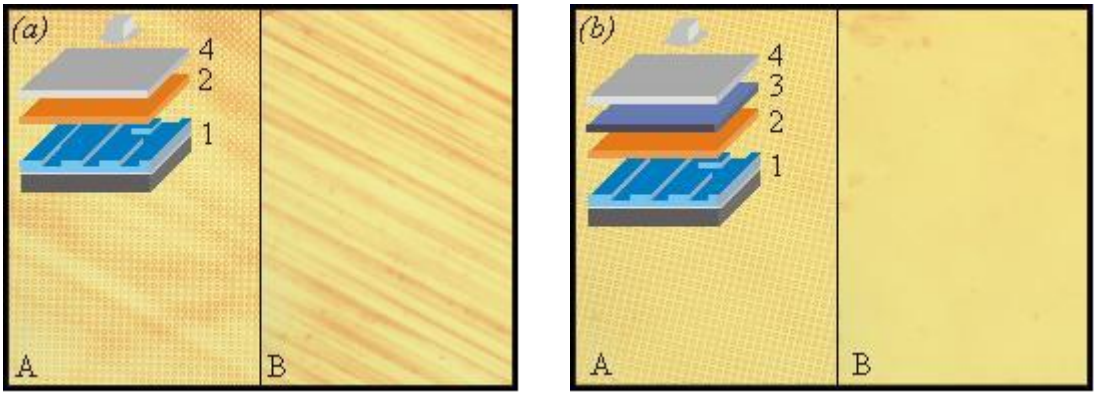


Figure 9. Elimination of backside patterning of 125 μm thick PMMA sheets through the use of a high T_g polymer layer. In (a) the PMMA (2) is only sandwiched between the mould (1) and the aluminium (4). In (b) the PMMA is protected by the high T_g polymer (3). In each case, the optical images show the surface

patterning by the mould on the front side of the polymer (A) and the presence (or absence) of the patterning by the aluminium on the reverse (B).

An example of the process graph for the nanoembossing is given in figure 10. The system is first warmed up using two heating steps, **A**. The polymer is then heated, **B**, to a temperature above that of the polymer T_g, causing the polymer to soften. Once the temperature has stabilised, the mould is forced into the surface of the polymer under pressure, **C**, which forces the polymer to fill the structures in the patterned mould. After a period of time the polymer fills the structures completely. The embossed polymer film is then cooled, **D**, to a temperature below that of T_g. This forces the polymer to adopt the shape imparted by the mould. Finally, the pressure is removed, **E**, and the polymer and the mould can be removed from the apparatus. After a short period of cooling (~5 min), outside the press system, the embossed polymer sheet can simply be manually demoulded from the surface of the mould. (Note: after the system is warmed up, prior to the first embossing step, the warm up steps, **A**, do not need to be repeated.)

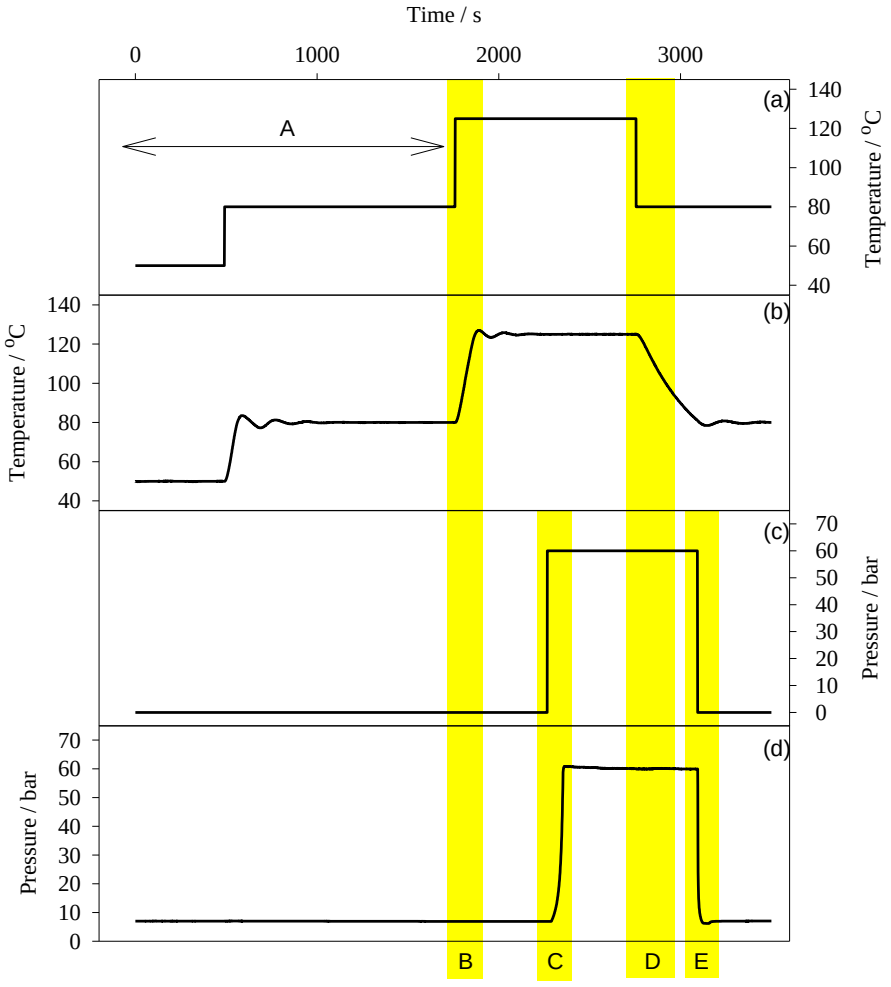
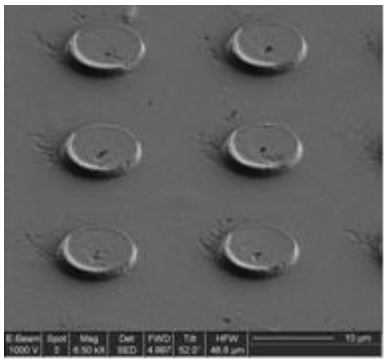


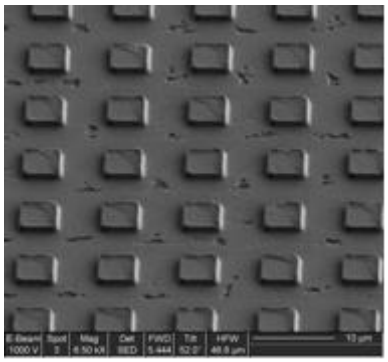
Figure 10. A typical example of a process graph for nanoembossing where (a) is the emperature set point, (b) is the recorded temperature, (c) is the pressure set point, and (d) is the recorded pressure (See text for details).

In this example, the total embossing time is ~20 minutes at 60 bar pressure. However, these process parameters can be reduced or increased depending on the type of polymer, the mould material and the dimensions of the structures to be produced. We have already applied this technique to freestanding polymer sheets of PMMA, PLA [100], and poly(ethylene naphthalate) (PEN) but it should be applicable to a wide range of thermoplastic polymers.

5.3 Polymer replica characterisation

PMMA replicas fabricated by embossing techniques have been characterised using some of the aforementioned characterisation methods. Imaging of the surfaces of the moulds and the patterned polymers has been achieved using optical microscopy, white light interferometry (Wyko NT110; Vecco Metrology, USA.), atomic force microscopy (Pico Plus; Molecular Imaging, USA), and scanning electron microscopy (SEM: Strata DB235; FEI Co., Netherlands.), depending on the size of the structures, or the substrate material, to be imaged. AFM measurements show that the pristine PMMA was found to have a typical surface roughness (r_a) of ~13 nm. Embossing of the polymer introduces structures into the surface of the polymer and consequently increases the roughness. An example of some regular microstructures embossed in PMMA is given in figure 11: examples of regular nanostructures embossed in PMMA are given in figure 12.





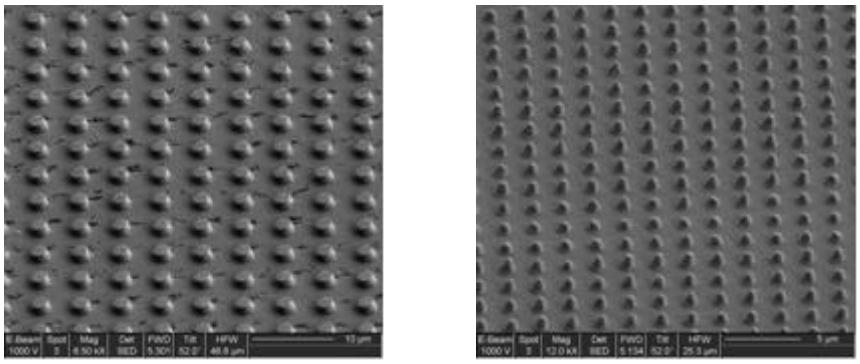


Figure 11. SEM images of regular microstructures, with dimensions of (a) 10 μm diameter, (b) 5 x 5 μm^2, (c) 2.5 μm diameter and (d) 1 μm diameter, and ~400 nm tall, embossed in a 125 μm thick freestanding PMMA sheet, over an area of 1 cm^2.

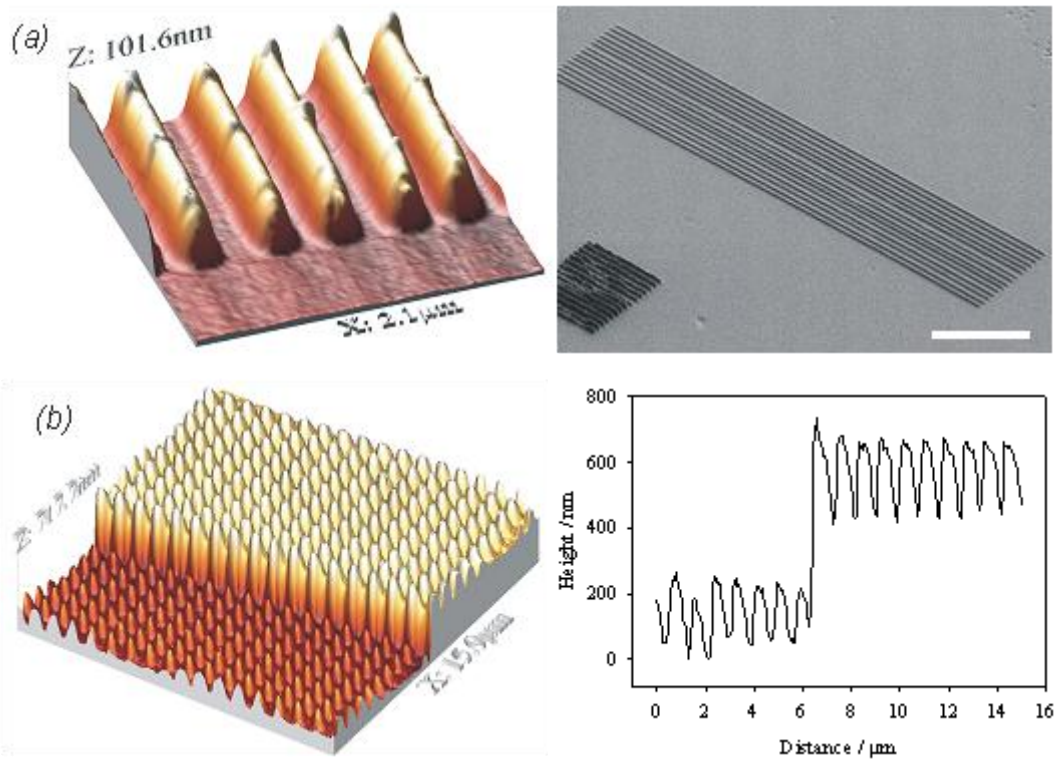


Figure 12. AFM and SEM images of regular nanostructures embossed in PMMA with dimensions of (a) 200 nm wide, 100 nm tall, 80 μm long lines, and (b), 200 nm tall, 1 μm diameter posts embossed on two levels of a 400 nm tall step. The aspect ratio of the lines in the bottom left hand corner of the SEM image in (a) was too high and hence they have collapsed [bar = 5 μm].

The wettability of the polymer surface was characterised using contact angle measurements both before and after embossing (Figure 13), and compared to soda lime silicate glass (commonly used in biological laboratories as microscope slides) and silicon nitride (the mould material). The advancing contact angles (water, 0.3 ml) give an idea of the hydrophobicity of each surface. The PMMA surface, when unstructured,

is slightly more hydrophilic than the silicon nitride, but less so than the glass. The advancing contact angle of PMMA is seen to increase from 73.4°, a value that agrees with previously published results [101, 102], to 74.7°, upon sterilisation with gamma radiation (see section 5.4). A similar small increase, from 75° to 81°, has been found after gamma irradiation of poly(caprolactone) (PCL) surfaces, and has been attributed to a slight oxidation of the surface via free radicals produced during the irradiation [103]. The advancing contact angles for the glass, 35.0°, and silicon nitride, 73.0°, also approach those recorded in the literature, i.e. 40° [104] and 85.9° [105] respectively. However in these cases, the hydrophobicity of the samples was seen to increase more dramatically (by more than 10° in each case). Again this may be due to oxidation of the surface after the irradiation and, as the surface material is more densely packed compared to the PMMA, the effect is more pronounced.

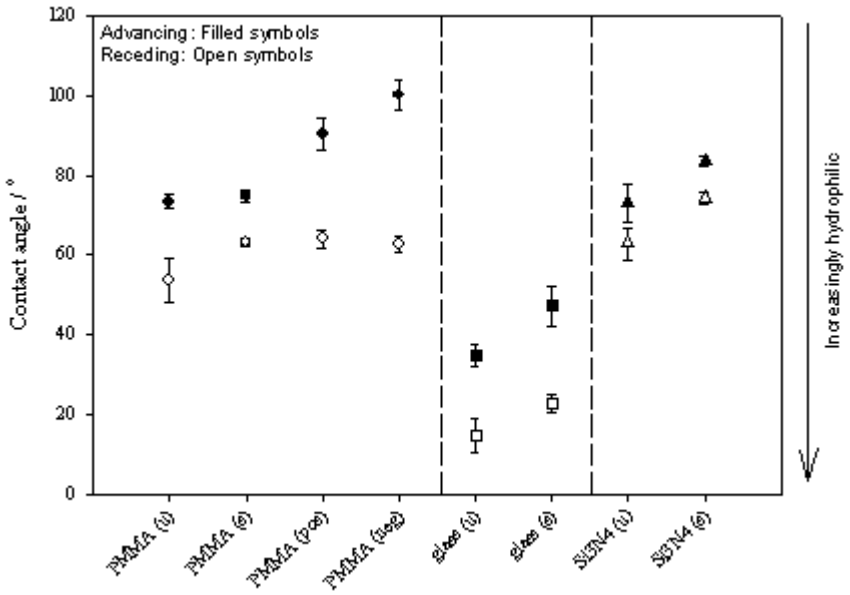


Figure 13. Advancing (filled symbols) and receding (open symbols) water (0.3 ml) contact angle measurements of PMMA surfaces: also shown for comparison are measurements for glass and silicon nitride (Si_3N_4).

Key: (u) unsterilised, non-structured; (s) sterilised, non-structured; (pos) sterilised, positively structured with 5 μm^2, 400 nm tall posts; (neg) sterilised, negatively structured with 5 μm^2, 400 nm deep holes.

Interestingly, upon microstructuring the PMMA, the surface becomes increasingly hydrophobic. This effect has also been observed for PEN, although in this case the contact angle was found to decrease upon structuring the polymer surface [99].

The contact angle hysteresis, the difference between the advancing and receding

contact angles, gives a value for the surface wettability, which in turn gives an idea of the roughness of the surface. Measuring the receding contact angle for the sterilized PMMA (63.2°) gives a H_2O contact angle hysteresis of ~12°, a value indicative of a low surface roughness, which is consistent with the result obtained through the AFM measurements. As expected, when the PMMA is microstructured, the hysteresis increases due to the increased surface roughness introduced onto the polymer surface by the structuring. Upon sterilisation, the hysteresis of the PMMA decreases by 9°, suggesting a decrease in surface roughness, possibly due to some local surface etching or melting [103]. Similarly, there is a slight increase in the hysteresis for the glass, suggesting an increase in surface roughness, but no change in the values for silicon nitride.

The optical transmission of pristine PMMA and PEN has been measured [99], in the range 300 to 800 nm, and compared with that of a 1.5 μm thick, soda lime silicate glass cover slip (Figure 14). PMMA is seen to have an optical transparency rivalling glass throughout the near IR/visible region of the electromagnetic spectrum. Although this transmittance decreases in the UV region of the spectrum, the polymer still transmits some 60% of the incident light at a wavelength of 300 nm. PEN in comparison transmits ~80% of the incident light in the near IR/visible region, but its transmission falls rapidly as the UV region is encountered at ~400 nm, due to the presence of the UV-adsorbing naphthalene moiety in the polymer matrix. The addition of structures to one surface of the polymer does decrease the transmittance (by as much as 20%), but the samples are still sufficiently transparent to be used with optical microscopes commonly found in a biological laboratory.

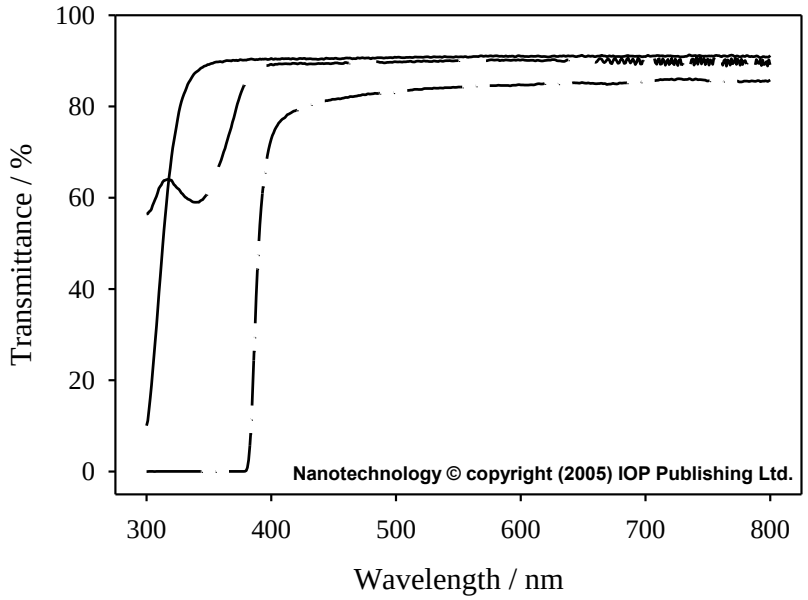


Figure 14. Optical transmission spectra of glass (solid curve), PMMA (dashed curve) and PEN (dash/dot curve) at wavelengths close to the visible region of the electromagnetic spectrum, showing the percentage of visible radiation transmission for each, compared to an air blank, and the near-UV absorption of each sample [99].

5.4 Sterilisation

To ensure the sterility of the polymers prior to use in in-vitro culturing experiments, a number of sterilisation techniques commonly used for the sterilisation of apparatus used in biological experiments, were investigated with varying degrees of success. Due to the low heat tolerance of the PMMA samples, the typical sterilisation temperatures (~120°C) used in an autoclave caused the PMMA to deform and caused the structures on the surface to melt. The low solvent tolerance of the PMMA caused similar effects when the PMMA was immersed in 70% ethanol (although the polymer showed no adverse effects after rinsing with propan-2-ol). To sterilise the PMMA samples we therefore turned to UV and gamma ray sterilisation. Gamma ray sterilisation, at 25 kGrays dose, was preferred due to the possibility of UV light affecting the chemical structure of the polymer. AFM and contact angle measurements confirmed that the surface roughness and wettability of the PMMA did not change, suggesting that the sterilisation did not affect the physical and chemical structure of the polymer surface to any great degree.

Although neither the ethanol immersion nor the autoclaving techniques were suitable for PMMA structured surfaces, they could be used with more robust polymers, such as PEN. A structured PEN film was initially cleaned and sterilised using gamma

radiation. The surface of the polymer was then used to culture MG63 osteoblast cell line for 7 days using an established technique [106]. After this, the PEN was thoroughly cleaned and re-sterilised by immersion in 70% ethanol. The sterilised PEN was then placed in culture medium in the absence of cells to examine whether any growth occurred. After a further 7 days, no osteoblast cell growth was observed and the chemical sterilisation was assumed to be successful. This suggests that PEN films can be re-used for culturing of MG63 cells as long as thorough sterilisation is performed between culturing experiments. The PEN has this advantage over PMMA due to its higher chemical resistivity. This allows it to be immersed in the 70% ethanol without degradation or deformation of the structures in the polymer surface.

5.5 Chemical functionalisation of polymer surfaces

Chemical functionalisation of the surface of PMMA substrates has also been achieved using a variety of SAM molecules. These have been further chemically modified to add such linking molecules as required for activation with biological moieties, or to change the surface properties. The functionalisation has been patterned by using microcontact printing [107]. For example, the PMMA surface was functionalised with a biotin pattern (Figure 15). The PMMA is first hydrolysed, **1**, and activated by the addition of PFP/EDC, **2**. The biotin pattern is then added via a PDMS stamp that has been immersed in Biotin-EZ (Culteck). The biotin only attaches to the surface in areas of mutual contact between the stamp and the surface, **3**. The areas not patterned with the biotin are then blocked using 2-(2-aminoethoxy)ethanol to avoid non specific adsroption in these areas, **4**. The pattern was imaged by exposing it to a solution of Texas red-labelled streptavidin. The streptavidin links to the biotin coated areas and the pattern can be imaged via fluorescence spectroscopy (Figure 16).

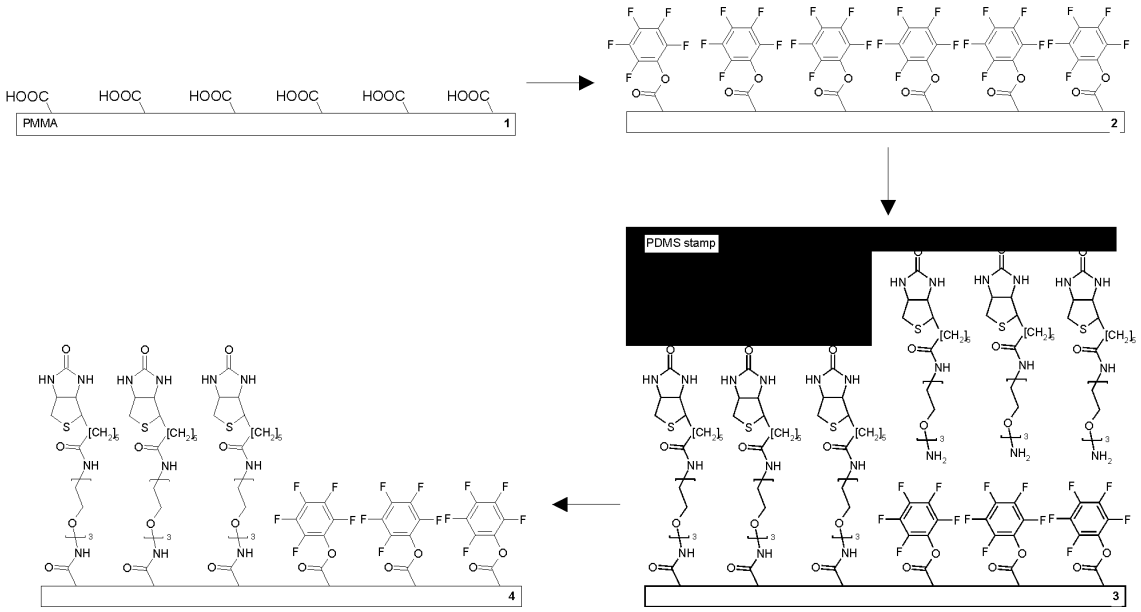


Figure 15. Scheme showing the selective patterning of a PMMA surface with biotin (see text for details).

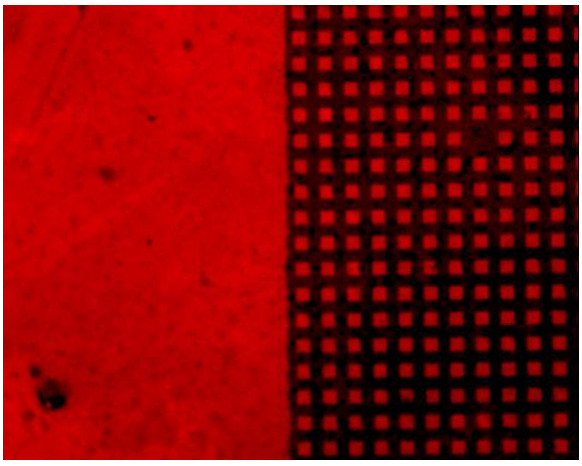


Figure 16. A PMMA surface functionalised with a biotin pattern using a microcontact printing technique. This pattern was subjected to Texas Red dye, and imaged using fluorescence microscopy.

5.6 Biomedical applications of patterned polymer surfaces

The first requirement of polymer substrates that are to be used for in-vitro cell-surface interactions is to withstand culture solutions and to support growth of the cells on the (structured) surface. Fortunately, in the first case, as most cell lines are sensitive to harsh environmental conditions, most culture solutions are based on aqueous solutions buffered to around physiological pH levels (i.e. ~pH 7 [108]). They are normally used at temperatures close to those found in the body (~37°C), and contain little more than dissolved salts and nutrients for cell consumption. Although many polymer systems can cope with such culturing conditions, biologists sometimes require

slightly more forceful conditions to mimic other environments; such as the more acidic environment found in gastric fluids, where the solution pH can be as low as pH 1 [109]; when attempting to use thermophilus or hyperthermophilus bacteria, which can have optimal growing temperatures of up to 100°C (e.g. the genus *Pyrodictum*) [110]; or during fermentation processes, where the temperature and solution pH can change with time.

It must also be remembered that the polymer should be able to withstand post-culturing processes that require it to withstand exacting environmental conditions, or to be inert with respect to chemical processes. Examples of the former can include preparation for certain microscopy techniques, such as scanning electron microscopy, where the cells are dried using carbon dioxide at its critical point (31°C and 7.4 kPa). Subsequently, the polymer used for the cell-substrate interactions must also be able to withstand this freezing process without deformation or disintegration. In the latter case, where the polymer must be inert to post-culture chemical treatments, examples include the staining techniques commonly used for examination of cell structure. Obviously, the polymer itself must not become stained, obscuring the structures to be observed in the staining experiment, nor must it directly interfere with the experiment, for example via an inherent fluorescence which would interfere with fluorescence experiments. Knowledge of the properties of polymers, to narrow down potential candidates for a particular experiment, allied to a systematic approach to testing the most likely candidates, should lead to a polymer that satisfies the structuring requirements and experimental tolerances of the experiment.

When structured polymer surfaces are required, PMMA is a good candidate due to its excellent structuring and optical properties. These properties allow for the imaging of cells through the bulk of the polymer, so that cell-surface interactions can be viewed directly from below, rather than through the bulk of the cell (Figure 17). However, where a higher thermal or chemical resistance is required, polymers such as PEN may be used. PEN is biocompatible and may be structured at the nanoscale [96], but it has a much higher resistance to acids and solvents than PMMA, and has a temperature tolerance some 100°C higher. Conversely, PEN contains a naphthalene group in its monomer unit, which causes it to absorb strongly in the ultraviolet region of the electromagnetic spectrum, and hence makes it likely to fluoresce in the visible region. Consequently, it is undesirable for fluorescence measurements as the polymer will produce a high background fluorescence signal.

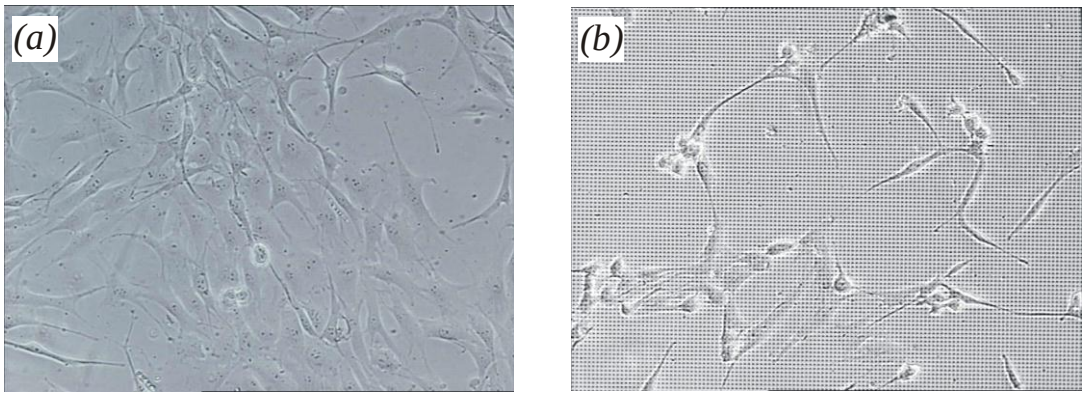


Figure 17. Optical microscope images of MG63 cells on the surface of (a) unstructured PMMA and (b) PMMA structured with 2.5 μm^2 posts imaged from below, through the bulk of the polymer, using an ordinary optical microscope (30x magnification).

The nanoembossing technique described here, for the production of micro/nanostructured polymer surfaces for cell-substrate interactions, has two major advantages over the traditional method for producing nanoembossed polymer surfaces deposited on inorganic substrates, as used in nanoimprint lithography. Firstly, the freestanding polymer sheet does away with the need for any bio-incompatible inorganic substrates that could affect cell culture. Further, such freestanding polymers, which have normally been purchased from commercial sources, have been fabricated some time before they are used. This is advantageous because polymers in general have the disadvantage that they tend to degas over time: i.e. gas or solvent molecules, which are trapped within the polymer matrix during the production stage, slowly escape from the polymer into the surrounding environment. Therefore, the older the polymer is when it is used for culture experiments, the less likely it is to have large quantities of trapped gasses, and hence the less likely it is to affect cell culturing. Secondly, the freestanding polymer can easily be cut to the required shape for use with existing cell culture apparatus, e.g. culture plates. The sheets can also be used with reusable culture plate systems (for example, the elastomeric polymer-based FlexiPERM®), either by immobilising a small piece of the polymer to a suitable surface, or by structuring the polymer so that the structured areas align with the culture wells.

Figure 18 shows conventional SEM images of a cell on a structured PMMA surface. The cell was cultured on the surface of the polymer then dried using CO_2. A thin coating of platinum was then applied to the sample to facilitate imaging by allowing a conducting pathway for electrons. The cell is seen to lie at an angle to the

pattern of structures on the surface of the polymer, and has fibrils protruding from its body helping to anchor it to the surface (Figure 18a). It has extended a microspike (towards the bottom right of the image) which it uses to explore its surroundings and was possibly in the act of moving to another area of the surface. This is suggested by the lack of fibrils at the back of the cell (toward the top of the image), compared to those at the front, and the presence of the microspike. The cell would have drawn in these protrusions from its rear end prior to moving, and would use the ones at the front to anchor itself as it moves forward [111]. Figure 18b shows a group of 4-5 cells on a nanostructured PMMA surface. The cells are seen to align with a pattern of 200 nm wide, 200 nm tall and 400 nm period line structures. Again, the cells nanosized fibrils can be clearly seen.

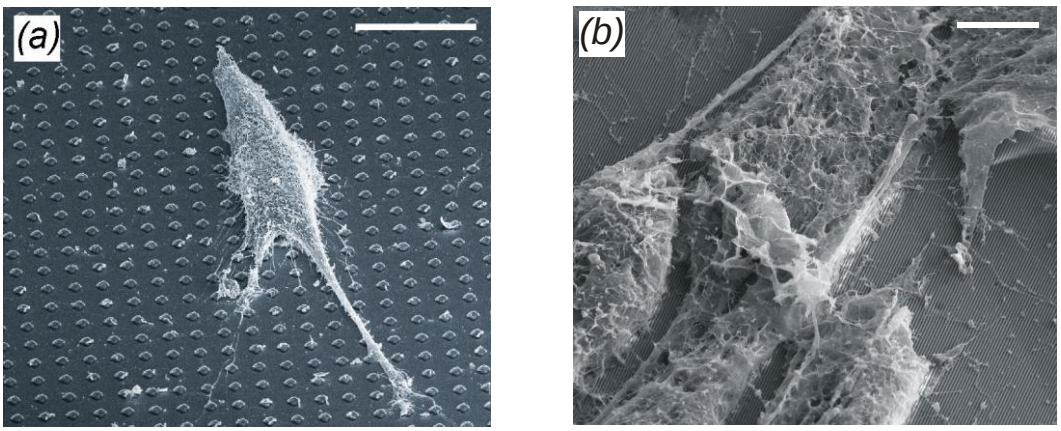


Figure 18. Scanning electron microscopy image of cells on (a) a microstructured PMMA surface [bar = 20 μm], and (b) a nanostructured PMMA surface [bar = 10 μm].

Figure 19 gives and example of images showing the interaction of osteoblast cells with freestanding, thin film PMMA surfaces that have been chemically modified with the previously described functionalisation technique (section 5.5). Confocal fluorescence microscopy has been used to examine the distribution of various structural components within the cell. In (a), actin within the cell is stained using Phalloidin-TRITC, and is shown in red. Integrin, a protein that is found at points where the cell attaches itself to a surface, is stained using Alexa Fluor 488, and is shown in green. The image shows that the actin is very much aligned along the length of the cell, in stress bundles, suggesting that there is a certain degree of stress in the cell, and that the integrin is concentrated into focal adhesion points, especially at the edge of the cell. In (b), the nucleus of the cell is stained using DAPI, and is coloured in blue, and

fibronectin, within the cell and patterned on the surface of the PMMA, is shown in red. The attachment points of the cell to the polymer in this case is seen to be affected by the position of the patterned fibronectin, with the attachment points preferring the fibronectin patterned areas to the pristine polymer.

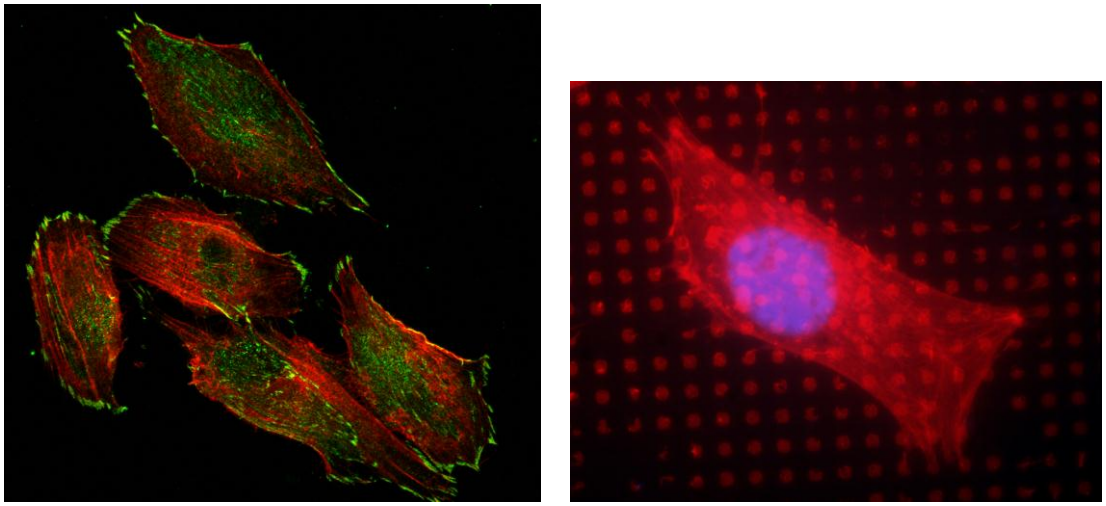


Figure 19. Osteoblasts on the surface of (a) an unstructured (20x magnification) and (b) a chemically structured (100x magnification) PMMA surface imaged using fluorescence confocal microscopy. In (a) the integrin (green) gives an idea of the attachment points of the cell to the surface, whereas in (b) the fibronectin (red), both patterned on the surface and within the cell, can be seen.
(Note that the colours are added electronically after the confocal fluorescence images are taken to aid in examination of the position of the proteins.)

6. CONCLUSION

The burgeoning field of biological cellular interaction studies requires a method for the production of a large number of replica surfaces containing micro- and nanoscale structures, preferably with thermal and chemical resistance. Typical engineering solutions to this problem, especially at the nanoscale, are dependent on serial techniques, or techniques that require a large number of fabrication steps. One possible parallel fabrication method that allows for the rapid production of micro/nanostructured surfaces is nanoimprint lithography. However, the normal nanoimprint lithography method, in which a (typically inorganic) substrate is required to support the polymer during structuring, limits the usefulness of this technique for biomedical applications, especially with respect to using existing apparatus for culturing and analysis.

We have presented a fabrication method, based on nanoimprint lithography, in which freestanding thin films of polymers can be structured at both the micro- and

nanoscales. These can be used in cell-surface interaction experiments using existing biological apparatus. The polymers used can be chosen depending on the necessary requirements for sterilisation, culturing and analysis techniques used in the experiment. The surface of the polymer can be functionalised using self assembled monolayers to alter the surface properties and, using techniques such as micro-contact printing, this functionalisation can be patterned to the required dimensions.

To date, we have examined only a small number of polymers that can be structured in this way. However, as this technique can produce topographically patterned surfaces quickly and easily, and the subsequent patterned chemical functionalisation of the surfaces is possible, a large number of polymer/functionalisation/cell combinations could be examined in a short space of time. Going further, block co-polymers could be used to present more than one polymer type for surface functionalisation.

Development of techniques for 3-D scaffold production with regular porosity will be of further benefit to cell culture experiments however these techniques will need to ensure rapid, defect-free production before being accepted for general use. To this end, a combination of surface structuring and suitable functionalisation and dicing techniques, could lead to the production of polymer-based, self assembling "building blocks". These building blocks could be used in the production of 3D structures on substrates which have a patterned functionalised surface for "guidance" of the initial fabrication stages. Such micro- or nano-building blocks could be made to align with the functionalised substrate, produce a multilayered "wall", and finally present pre-prepared bioactive surfaces to cells in three dimensions.

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