Chapter 6
Surface Modification of Tissue Engineering Scaffolds

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Abstract Scaffolds in tissue engineering provide a substrate for cells to grow on, in order to form functional, organised tissue. The ideal scaffold thus possesses mechanical properties to cope with physiological loads, degradation profiles to match the rate of tissue regeneration, while also eliciting favourable host responses. There is, however, often a trade-off between having optimal bulk or surface properties. To address this, various strategies to perform surface modification have been developed to tailor scaffolds for specific applications. These strategies are discussed in this chapter and may be broadly categorised under modification based largely on physical mechanisms (employed largely to induce changes in topography, roughness or wettability) or chemical modification (employed largely to introduce new functional groups on a surface). Subsequently, the characterisation of the modified surface is necessary, in order to facilitate design for use as scaffolds. These evaluations are similarly discussed in this chapter as physical, chemical and biological characterisation methods. The latter, in particular, is unique to materials used in medical applications (including tissue engineering scaffolds) and the section discussed the use of the ISO 10993 set of standards.

Keywords Surface modification · Scaffolds · Tissue engineering · Surface characterisation · Biocompatibility

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6.1 Introduction

A wide range of biomaterials, both synthetic and biologically derived, are used widely in biomedical applications, including utility as scaffolds in tissue engineering. These materials are largely selected on their bulk properties, such as mechanical strength and degradation properties, in order to meet structural requirements [1, 2]. However, host responses are largely mediated by interactions with the material surfaces [3], and there emerges a need to tailor these surface properties to elicit appropriate biological responses, while retaining the bulk properties on which the materials were selected [4]. To meet these needs, much research has been dedicated toward the development of surface modification technologies. This chapter discusses the common technologies being used, as well as some methods employed to characterise the modified surfaces.

6.2 Surface Modification Techniques

6.2.1 Physical Surface Modification

The focus of biomaterials is shifting from bioinert implants to bioactive designs, in order to manipulate the interactions between cell physiologic systems and material properties, including physical cues. Physical surface modification refers to processes that apply physical methods to effect change in physical properties (such as roughness and wettability), biochemical properties (biochemical components, functional groups and/or the distribution of them) or topographic structure (lattice structure, pore size and micropatterns) of the surface. Through the physical, biochemical or topographic cues conferred by these modification methods, the adhesion, proliferation, alignment and intracellular physiological activities of cells on modified surface can be controlled. More recently, physical modification methods have also been shown to elicit antimicrobial effects [5, 6] and even retard blood coagulation [7–9].

6.2.1.1 Topographical Engineering

Scaffolds in tissue engineering are analogous to the extracellular matrix (ECM) in that they provide the mechanical substrate for cell growth. Besides structural support, it has become evident that physical cues, in the form of topographical microstructures, are capable of guiding cell alignment and migration in the microenvironment [10, 11]. This phenomenon was described in as early as 1912 by Harrison in the direction of cell motion on spider web and was later defined as ‘contact guidance’ by Weiss [12, 13]. Presently, biological reactions triggered by biomaterial topography have been demonstrated on parenchymal cells, inflammatory cells and bacteria.

Cell Attachment, Growth, Morphogenesis and Differentiation In earlier studies, fibroblasts seeded on quartz slides with parallel ridge-groove structures showed alignment and elongation along the direction of gratings, reflecting contact guidance. Such effects have been shown to be dependent on topographical parameters, such as groove depth [14]. In a study on PMMA substrates with similar patterns, larger depth and width were found to be effective in restricting the lateral movement of fibroblasts across groove structures, smaller widths restricted the longitudinal movement along the ridges [15]. These observations indicate that cells can recognise the dimension of a surface topography both at microscales (cellular sizes) and nanoscales (near the sizes of filopodia and lamellipodia) [16], believed to be mediated by patterning of focal adhesions and filopodial sensing [17, 18]. Focal adhesions (FA) are multi-protein complexes mechanically linking intracellular actin to extracellular substrates via integrin-ligand bundles. It has been evidenced from multiple studies that matured FA result in elongated morphology, aligned in the direction of actin filaments and subject to forces correlating with the main axis of FA elongation [19], and cells adhered on the interface of dual microstructured films have been demonstrated to acquire 'half-cell' alignments [20]. These highlight the utility of topographical engineering in generating the specific anisotropy found in connective, mechano-sensitive, electro-active and shear-responsive tissues [16].

Topographic cues also have mechanical effects on cells by causing deformation of cytoskeleton and adjusting intracellular tension, with accordant changes in nuclear structure, epigenetic signals and expression profiles. These biochemical signals and mechanical signals may in turn further modulate cellular responses and influence cell physiological activities in a cascade of events, influencing cell motility, apoptosis [21], proliferation [22, 23] and differentiation [23–26]. Additionally, the formed focal adhesions serve as biochemical signal sensors to allow transmembrane signal transduction, such as focal adhesion kinase pathways, by activation of integrin receptors [27–29]. Wang et al. reported significant up-regulation of myogenic genes in human mesenchymal stem cells (hMSCs) [24, 25] and tenogenic genes in human tendon cells [26, 30], when these cells were cultured on anisotropic poly(ε-caprolactone) (PCL) surfaces with aligned topographies. In nerve regeneration applications, up-regulation of neural markers at mRNA and protein levels was observed in hMSCs on aligned PCL nanofibre scaffold when compared with that on polystyrene (PS) plate, indicating an enhanced commitment of MSCs into neural cells [31]. Considering, however, that elongated morphologies may have concomitant downstream effects [26] and noting that gene expression triggered by topographies lack tissue specificity [23–25, 32], it would be too simplistic to assume that topographical engineering (or any other single approach) can be used in isolation in therapeutic tissue regeneration.

Immuno-Regulation While generally an important factor in biocompatibility, the immune response is particularly critical in determining the long-term outcome of implants, through the mediation of host responses such as chronic inflammation, fibrosis or integration. For example, neutrophils and macrophages serve both phagocytic and signalling roles, and materials with defined surface structural and

topographical features were reported to favourably modulate the innate immune response, leading to improved healing outcomes. TiO_2 honeycomb-like structures at a minimal scale of 90 nm were reported to facilitate macrophage filopodia formation and up-regulate the Rho family of guanosine triphosphatases (*RhoA*, *Rac1* and *CDC42*), in turn reinforcing the polarisation of macrophages through the activation of the RhoA/Rho-associated protein kinase signalling pathway [33] Similarly, osseointegration events on controlled nanotopographical structures were found to be heavily influenced by microscale features and nanopatterns on implant in vivo, through modulation of inflammatory responses [34]. In a study on breast implants with different surface topographies (average roughness from 0 to 90 μm) in mice and rabbits, an average roughness of 4 μm was found to result in the least amount of inflammation and foreign body response [35]. These observations may be due in part to macrophage polarisation, as demonstrated by Wang et al. [36]. In the latter, the nanotopography of hydroxyapatite disks was shown to influence tissue inflammation, up-regulate gene expression of M2 phenotypic marker and raise the fraction of ARG+ M2 macrophages in vivo. Such findings may be extended to applications beyond implants for tissue engineering, and more generally to modulating immune responses in vivo. In a recent study, heparin-doped polypyrrole (PPy/Hep) electrodes of different surface roughness, with surface roughness values from 5.5 to 17.6 nm, demonstrated different degrees of macrophage recruitment, inflammatory polarisation and fibrotic tissue formation, and could successfully record electrocardiographic signals for up to 10 days without substantial decreases in sensitivity [37]. Such studies indicate that an improved understanding of the relationship between material features and its immunomodulatory potential may help in the design of implantable materials.

Anti-bacterial Effects Following colonisation by bacteria, the formation of a biofilm is typically detrimental for medical devices; endotoxic effects aside, biofilms formed also impede the device performance. It has been shown that bacteria adhere preferentially to topographies that maximise their contact area to surface [38], in accordance with adhesion point theories [39]. When organisms are smaller than topographical structures, the available contact area for bacteria is large; thus, it is able to obtain adhesive strength. Secondarily, the stiffness of a cell wall limits the ability of bacteria to adapt freely to the surface topographies at very small sizes [40]. Such reasoning is supported by studies showing bacteria prefer to adhere at square corners, convex features rather than on flat or concave walls. Yang et al. [41] have proposed a contact-based effect involving energetically favourable adhesion sites and physical confinement. The preferential adhesion points can also influence bacteria motility, thereby interfering with bacterial adhesion. Specifically, surface topographies with line structures decrease bacteria attachment compared to flat surfaces [42], modulated by the elongated morphology and up-regulation of flagellar genes. Meanwhile, bacteria deposits on different surface topographies may confer mechanical stretch to the bacterial cell surface. This can cause the rupture of bacterial cell membrane, leading to bacteria lysis and death. Such bactericidal properties have also been described on pillar topographies, regardless of material surface chemistry.

The killing effect can be enhanced further by multiscale roughness, for example, nano- and microstructures for a highly biocide-free bactericidal property [43].

Physical Modification Methods In order to modify the topographical structure of tissue engineering scaffold, materials are often either added onto substrate surface by methods like nanofibre coating [23, 44] and plasma deposition [45] or ablated by methods like chemical etching and laser corrosion [46, 47]. Li et al. modified the surface of a flexible PCL film with fibres and demonstrated improved deposition efficacy on rougher surfaces [44]. A further study by Guo et al. reported post-fabrication processing by single-axial drawing that induced a coating of highly aligned fibrous topography, leading to enhanced adhesion to substrate and possible application in rotator cuff tendon repair [23, 30]. Similarly, mechanical techniques like stretching may also be applied directly to polymer materials to avoid weight loss and change in material composition while creating new microstructures. After uniaxial stretching, for example, hierarchically and heterologously oriented ridge-groove structures that mimicked ECM more are observed on PCL films and successfully guided human bone marrow MSC elongation and alignment with a preferential orientation determined by the topographical anisotropy [24]. The elongation and alignment of MSC could be observed as soon as 12 h post seeding and demonstrated to last as long as 15 days in vitro. More specifically, altered cell morphology, cytoskeletal reorientation and nucleus elongation were pointed out in the following studies, with increased expression of myogenic genes in MSCs [24, 25] and tenogenic genes in tenocytes [26] following extended culture on the stretched PCL films. The topographical features of stretched PCL films can be further controlled by alkaline hydrolysis [46]. Small concaved features formed at the edge of ridges after soaking in aqueous NaOH solution for longer than 10 days that developed into parallel grooves across the ridges and finally split the ridges into small parallel islands after 30 days of soaking. With declined ridge height and aspect ratio between ridges and grooves resulted from hydrolysis, the guidance effect of topographies on MSC alignment and elongation may be compromised. In addition, the ridge-groove topographies can be further augmented with femtosecond laser microperforation to create secondary microfeatures [20, 32]. Thus, engineered scaffolds were used to generate hybrid cell-material sheet, for example, when MSCs and human umbilical vein endothelial cells were seeded separately on each face of the stretched PCL film. This construct allowed heterotypic cell-cell contacts across the film and mimicked the myoendothelial communication between tunica media and intima. In a recent study, Luo et al. applied a femtosecond laser to write micro-grooves on PCL film, where laser engineering was found to influence both surface wettability and 3D cell morphology [47]. Even with precise laser engineering, physical alterations are coupled with chemical changes on the biomaterials surface (such as polymerisation and chain scission), and these effects must be considered in rational design and selection of processing methods.

6.2.1.2 Wettability Engineering

Wettability refers to the interaction at the two-phase interface between fluid and solid. Solid surface with a greater wettability is more favourable for the fluid to spread over or adhere to it, so that the contact angle between solid–fluid interface and vapour–fluid interface is smaller. Wettability presents as one of the primary concerns in scaffold material design, for its influence on both initial cell attachment and migration on scaffold surfaces via adsorption of proteins from culture medium in vitro or from extracellular fluid in vivo and then binding to cell adhesion molecules on cell surface. The preferable range of wettability varies according to the kind of cells. For example, a range of water contact angles for cell adhesion and growth was suggested to be from 50° to 60° [48], but a more hydrophilic surface with the water contact angle between 20° and 40° was more suitable for cell attachment of NIH 3T3 fibroblasts [49]. When inherent surface wettability is not favourable for cell growth, surface modification may be necessary. For example, PCL fibres from electrospinning are considered too hydrophobic for direct cell adhesion, with a typical water contact angle of 100–130° [23]. By depositing the PCL fibres on a substrate, subjecting to single-axial drawing, or blending with bioactive tricalcium phosphate particles, the contact angles of the fibres can be further adjusted. Coating with polymers such as poly(vinyl phosphonic acid-*co*-acrylic acid) (PVPA), can also render a surface hydrophilic, with water contact angles dropping to 43.3 ± 1.2° [50]. After 14 days in vitro culture, osteoblasts on PCL/PVPA scaffold generated a better-defined cytoskeleton than those on uncoated PCL scaffold, indicating a better cell spreading due to improved wettability of PCL fibre. Such methods reflect the classical approach of modifying the surface of a bulk material that possesses desirable physical properties, in order to confer the preferred properties of the surface material (which typically has inadequate bulk properties).

Alkaline hydrolysis is one of the mature techniques to modify surface wettability for polyester biomaterials such as PCL [51], PLA [52, 53] and PLGA [54, 55] and already has been applied in industry. After treatment with alkaline agent (usually mild NaOH solution), the ester bonds on the surface of polyester materials break and form carboxyl and hydroxyl end-groups. As observed in the previous section, hydrolysis may also alter topographies of the surface, resulting in an improved roughness. The increased surface energy results in greater affinity to water molecules, resulting in the higher hydrophilicity favoured by specific cell types. More controlled and targeted approaches using laser-assisted techniques have been well investigated to modify biomaterial surface wettability by making the controllable topographical roughness for implants and bioelectronic applications [16, 56]. Based on the Wenzel equation, the increase in roughness of a solid surface can either increase the hydrophilicity in a hydrophilic system in which the water contact angle is smaller than 90° or increase the hydrophobicity in a hydrophobic system where the water contact angle is larger than 90°. Thus, by changing the surface roughness, the wettability of biomaterials can be optimised for a better cytocompatibility. Extreme ultraviolet (EUV) irradiation is another approach for roughness optimisation for

polymeric materials. For instance, polyether ether ketone (PEEK) commonly used in reconstructive surgery has unique mechanical and physicochemical properties, but lack polar surface chemical groups, and has an inherently low surface energy. To modify extremely stable materials such as the PEEK surface, EUV irradiation in the presence of oxygen and nitrogen gases were used [57], which made significant changes to surface topography with increased surface roughness, formation of conical structures and incorporation of nitrogen and oxygen atoms. As a result, the PEEK surface demonstrated non-cytotoxic properties and an enhancement in adhesion of human osteoblast-like MG63. Nanofibre deposition or nanoparticle deposition onto the surface of biomaterials is another approach to change surface roughness [44]. On PLA films incorporated with magnetic nanoparticles, enhanced adhesion and proliferation of cardiac-like rat myoblasts H9c2 was found on the film with the highest amount of embedded nanoparticles and hence the largest surface roughness with the largest water contact angle [58]. It is noteworthy that in this study, with the increase of surface roughness, film surface turned from being hydrophilic to hydrophobic, which looks contrary to abovementioned conclusion from the Wenzel equation. This is because surface wettability is influenced by complex factors including not only the roughness but also other aspects like electrical and chemical nature, which should also be taken into account at the design of material surface.

Other techniques for surface roughening are still under exploration. Borrowing ideas from micromanipulation, carving the surface of polymer materials or moving polymer chains at a microlevel and even nano-level high precision for surface modification may be realised under two-photon polymerisation (TPP), atomic force microscope (AFM) or scanning tunnelling microscope (STM) with manipulator and tip of scanning probe. As yet, such approaches are limited by high costs and availability of such facilities; these are further exacerbated by the demand for specialised cross-linking agents and material systems for TPP techniques, while only electrically conductive polymers can be processed under STM.

6.2.1.3 Physical Deposition

Physical deposition is a commonly-employed method to produce a functional coating layer on substrate material so as to grant the material with more desirable surface properties. In particular, bioactive components including inorganic particles, synthetic polymers, lipids, polysaccharides, peptides, proteins as well as cell receptor ligands [59, 60] are deposited onto scaffold materials for enhanced initial cell attachment and proliferation or to regulate intracellular protein synthesis and induce cell differentiation [61]. For the combination of functional materials with substrates, weak forces such as van der Waals forces, hydrogen bond force and electrostatic attraction force are formed during deposition. Based on the existing form of coating materials, physical deposition can be classified into solution deposition, vapour and sputtering deposition. The last two share a similar process that deposition of a material starts as a solid and transports to the substrate surface to build up a film slowly. Compared to chemical conjugation, physical modification might be less

stable which leads to gradual loss of the coating layer [62]. On the other hand, physical deposition has a broader application with the multitude of materials as substrate or as coating layer regardless of their chemical composition.

Solution deposition methods may be further subcategorised into immersion and adsorption, casting, dip coating and electrophoretic deposition. These methods are based on allowing the substrate to contact and adsorb the functional molecules dissolved in a liquid phase, followed by removal of the solvent by evaporation. Immersion and adsorption are the simplest methods by which substrate stands in a solution and spontaneously adsorbs the functional molecules uniformly dispersed in this solution. In applied electric field, deposition of coating molecules can be accelerated by electrophoretic motion of these molecules in solution toward the substrate placed at a corresponding electrode. This technique for physical deposition is named as electrophoretic deposition. Besides, the thickness of coating layer is determined by the time length of deposition and/or electric field strength. The technique as casting for modification of smooth and flat surface is developed from the same principle as immersion. Other than a static contact between substrate and solution, solution containing functional molecules is sprayed onto the substrate and subsequently spreads over the surface at high-speed spinning and thereby forms a thin liquid layer that leaves the functional molecules as a film covering the substrate after evaporation. The thickness of this coating film is controllable depending on both the speed of centrifuging and the viscosity of the solution. Dip coating is another popular low-cost technique for deposition on monolithic three-dimensional scaffolds. The process is completed by partially or fully inserting the substrate into coating solution followed by removal from the solution. For example, polypropylene (PP) has a remarkably low surface energy and poor surface functions [63]. By dip-coating of the PP surface with functional molecules, the surface segregation of these moieties yields a low-fouling surface. In addition, the process of dip coating can be repeated to obtain multiple coating layers, which is named as ‘layer-by-layer’ fabrication. Unlike simple immersing or spin casting, dip coating enables oriented alignment of coating layers formed by amphiphilic molecules like phospholipid and further affects surface properties of scaffold materials. Amphiphilic molecules refer to the kind of molecules that possesses both hydrophilic groups and lipophilic groups. In a solution, these molecules float on the surface of the solvent, keep the part of the molecule compatible with the solvent under liquid level and expose the other part above liquid level. Through different operating procedures consisted of dipping and removing, specific moieties on the amphiphilic molecules can be connected to the substrate surface or the previous coating layer. Especially on polymer substrates, entrapment of coating molecules in the surface may occur at the same time with physical adsorption due to the space created at swelling of polymers in the solvent, resulting in added amount of coating molecules deposited on polymer substrate [64].

Physical vapour deposition (PVD) is a surface coating method in which the coating material is vaporised in a cell at high temperature, often in the presence of gaseous plasma. The vapour is subsequently transported to the substrate surface and condenses to generate a thin film on it. Based on the methods applied to generate and

deposit material, PVD is further classified into vacuum evaporation deposition [65] pulsed laser deposition [66], electron beam deposition [67], cathodic arc deposition [68] and (most commonly for tissue engineering scaffolds) sputtering deposition [69]. In order to create gaseous coating material, inert gas (typically argon) is transferred into plasma and accelerated under a high-voltage electric field. Bombardment from this high-energy plasma at cathodic target frees coating molecules from solid source into a vapour phase through momentum transfer. These active molecules deposit on all surfaces inside a chamber to release energy and rebuild thermodynamic equilibrium, therefore forming a thin film on the substrate surface. Compared to evaporation deposition, sputtering deposition is superior in surface coating, being amenable to materials with relatively low melting points and forming a more sturdy coating on the substrate.

6.2.2 Chemical Modification

While biomaterials are primarily selected on their bulk properties, host responses are largely governed by the cell-material interactions at interface. It follows that synthetic materials, ranging from polymers to ceramics and metals, usually lack appropriate biological surface cues to elicit or direct desirable cellular and tissue responses such as adhesion, proliferation and immune response [1, 70–72]. To this end, chemical surface modifications may be performed to introduce biochemical cues onto material surfaces, while retaining the existing bulk material properties. These modification methods are summarised in Table 6.1, of which plasma-, gamma-, UV-, hydrolysis- and aminolysis-induced chemical modifications are the most common and are discussed in further detail.

6.2.2.1 Plasma-Induced Modification

Plasma-induced modification is useful for the selective creation of chemistry and topography on biomaterial surface with an excellent retention of the bulk

Table 6.1 Physical and chemical surface modification methods

Methods	References
Radiation (electron beam and gamma)	[73, 74]
Plasma (RF, microwave, acoustic, corona discharge)	[75–77]
Photo (UV and visible sources)	[78]
Ion beam (sputtering, etching, implantation)	[79, 80]
Gas phase deposition	[71, 81, 82]
Silanisation	[83]
Coating (with or without covalent bonding)	[84, 85]
Chemical reaction (oxidation, reduction, hydrolysis, aminolysis)	[70, 86, 87]

characteristics for specific biomedical applications [88–91]. Plasma, the fourth state of matter after solid, liquid and gas, is typically generated by applying high voltages to gases, under which the gas molecules or atoms will be ionised by the electrical discharge and therefore split up into electrons and ions [92]. The effectiveness of the ionisation process is dependent on operating parameters such as gas flow rate, pressure and constituents, as well as the distance between the discharge electrodes. Plasma can be further characterised with an energy distribution in the range of 10–20 eV to effectively modify most materials. The interaction between ionised gas species with surface substrates contacted in plasma could produce tailored physical and chemical modifications on substrate surface through ionic activity in plasma and functionalities (functional groups or free radicals) formation on the surface. As described earlier, in physical modification, the bombardment of ionic species powered by an electrical field can increase substrate surface roughness and promote interfacial adhesion of depositions [89]. Additionally, chemical modification can be achieved by controlling the (1) functional groups or free radicals which are generated on substrate surfaces by interaction between charged particles and surface molecules of the substrate [75] and (2) desirable monomer polymerisation and deposition on the surface of the substrate [93]. The functionalities formation on substrate surfaces may be altered by proper selection of the nature of the gaseous medium in plasma. Plasma generated in oxygen, ammonia and carbon dioxide gases can be used to introduce functionalities such as hydroperoxide, amino and carboxylic groups on substrate surface, respectively. Additionally, inert gases such as argon lead to the generation of free radicals on the polymer backbone, which are transformed into hydroperoxide bridges in the presence of oxygen and water vapour [1, 94]. Biomaterials with functionalities formation on their surface are either directly used for biomedical applications or continuously conjugated with following various desirable molecules for specific biomedical applications while those functionalities will be used as anchorage points. It has been reported that polyvinylidene fluoride membrane exposed to plasma resulted in grafting of quaternary ammonium compounds successfully [95]. This was performed via electron transfer for atom-transfer radical-polymerization; the thus-modified surface demonstrated a high inhibition rate ~98.3% of *E. coli* and ~98.5% of *S. aureus*, respectively. Control of plasma parameters and conjugants provides great versatility in the tailoring and customisation of surfaces of biomaterials. It is important to note here the process of plasma surface modification often leads to the formation of a layer of polymer on the surface and is coupled with physical alterations to the topography (discussed earlier in the preceding section) [1, 96, 97].

PCL and poly(lactide-*co*-caprolactone) (PLCL) have been used as bioresorbable polymers in numerous bioelectronics [95], medical devices [98] as well as for tissue engineering applications [23, 26, 30]. Biomolecule such as monomeric acrylic acid was conjugated on the surface of PCL and PLCL to optimise their bioactivities through plasma-induced surface modification [1, 75]. The exposure to argon/oxygen plasma under a UV irradiation resulted in formation of peroxide and hydroperoxide groups, which further initiated the addition polymerisation of acrylic acid to the PCL and PLCL membranes by decomposition of hydroperoxides. The degree of

polymerisation of acrylic acid on membrane surfaces is considerably influenced by the plasma exposure parameters, such as plasma power, pressure, exposure time and the reaction conditions involving monomer concentration and reaction time. Collagen and Jagged-1 peptides were then immobilised on the modified PCL and PLCL surface through carbodiimide coupling.

These technologies may be readily adapted toward other biomolecules, in order to tailor specific responses. Hyaluronic acid (HA), for example, is a biopolymer possessing numerous functions to be involved in wound repair, cell migration and cell signalling within the body [99, 100]. It is largely considered to be non-toxic, non-immunogenic, enzymatically degradable and relatively non-adhesive to cells and proteins [101]. Additionally, HA is involved in several physiological processes, including angiogenesis, extracellular matrix homeostasis, wound healing and the mediation of long-term inflammation. This versatile nature of HA has led to many studies not only on the preparation of HA alone but also on the subsequent usage for surface modification of biomaterials for specific medical applications. While HA can be applied as a physical coating, they get displaced easily, and covalent bonding of HA is necessary for use in biomedical applications. For example, HA covalently bonded on polydimethylsiloxane surface through oxygen plasma surface modification resulted in the decrease in the protein adsorption and significant cell growth and neural differentiation [96]; biomolecular binding to HA can further modulate biological activity, particularly for wound healing applications.

In performing plasma modifications, several parameters may be controlled in order to maximise polymerisation yield. Plasma treatment time, for example, significantly influences the formation of free radicals, and titration may be performed to establish the optimum plasma treatment time required for maximal free radical generation. Overexposure may also lead to the loss of free radicals that are otherwise responsible for peroxidation during exposure to oxygen [75, 102]. Additionally, the polymerisation conditions, such as spacer-monomer concentration, have profound impact on the yield of polymerisation. Without UV irradiation (typically at elevated temperatures), the yield initially increases with monomer concentration, reaches a maximum and then tends to decrease beyond a critical concentration [76], whereby extensive autocatalysis leads to homopolymerisation of the solution phase. In contrast, with UV irradiation and controlled temperatures, the yield typically increases continuously with the increase of the monomer concentration [1].

6.2.2.2 Ultraviolet (UV)-Induced Modification

UV irradiation is a simple, efficient and economic method widely used for surface modification of biomaterials [103, 104]. UV light is generally classified in four sub-bands: UVA (315–400 nm), UVB (280–315 nm), UVC (100–280 nm) and EUV (10–124 nm). At the wavelengths from 180 to 400 nm, UV light provides sufficient energy to disrupt molecular bonds on biomaterial surfaces, leading to a series of photo-physical, thermal and photochemical processes. However, this influence is often not limited only to superficial layer of the material but potentially alters the

material bulk properties [105]. As such, initiators are commonly used, which can reduce the dosage of UV irradiation in the surface modification process [91, 106, 107].

UV light irradiation has been extensively studied for surface modification of low- (LDPE) and high-density (HDPE) polyethylene membranes. For example, when HDPE was treated with selected active compounds and a photo-initiator under 254 nm UV excitation, the surface chemistry of HDPE was altered [107]. Functional moieties conjugated on the surface of HDPE via specific bonds resulted in increased wettability of the innately hydrophobic HDPE surface. However, the surface modification by UV irradiation could induce photodegradation and aging effect on the bulk polymer [108, 109].

In another example of UV-induced surface modification, irradiation of polyethylene terephthalate (PET) in formation of nano- and microstructures on the polymer surfaces, leading to more hydrophobic surfaces [110]. In contrast, when PET films were functionalised through conjugation with both RGD peptide and galactose ligands, enhanced cell adhesion and synergistic functions were also observed [91]. Plasma and UV irradiation can also be combined, by first irradiating the PET films with argon plasma (at power output of 40 W for 1 min) and then exposure to air (for 10 min) to induce the formation of peroxides and hydroperoxides on its surface. This is followed by UV irradiation to induce surface polymerisation of degassed monomeric solutions. Alternatively, this step could be replaced by the addition of some agents such as sodium periodate, which helps in oxygen depletion to ensure polymerisation efficiently. For example, exposure to UV (365 nm) may be performed to initiate the formation of a poly(acrylic acid) (pAAc) on the PET surface. Subsequently, RGD peptide and galactose ligands can be coupled to the pAAc layer using carbodiimide chemistry. This approach led to successful grafting of pAAc on PET films, with carboxyl-group density of 78.57 nmol/cm^2 available for subsequent conjugation of RGD peptides and galactose ligands.

More recently, extreme UV (EUV) radiation has been used as a source of high-energy ultraviolet radiation. The main advantage of the EUV irradiation is preservation of bulk properties of irradiated material due to its photon energy which is capable of breaking more molecular bonds at the upper surface of the material as compared to common UV light [111]. For the same reason, however, EUV radiation propagates only in vacuum, and hence, irradiation of materials in gaseous environment requires a special arrangement. Similar to plasma etching, EUV radiation is also used to produce nano-/microsized pattern on the surface of polymers [112].

6.2.2.3 Gamma-Induced Modification

Gamma radiation is an extremely high-frequency electromagnetic radiation and comprises high-energy photons generally above 110 keV. Gamma-induced modification is a well-established technique to modify biomaterial properties by gamma ray irradiation-induced modifications (grafting, cross-linking or gel formation). Cobalt-60 and cesium-137 are common sources used in gamma-induced

modifications. This technique has been intensively used for applications in the medical field for surface modification of materials to control blood-material interactions and conjugation of molecules in polymeric matrices to form specific chemical moieties or drug carriers. The major advantages of gamma-induced modification are as follows: (1) Due to its high-energy nature, initiators are not required in the process. Therefore, the purity of products may be maintained, as free radicals are formed on the polymer/monomer backbone. (2) Deep penetration of gamma rays through the polymer matrix enables rapid and uniform generation of free radicals and therefore could initiate the modification process throughout the entire material. (3) The gamma-induced modification can be performed at room temperatures. (4) It generates less environmental pollution than chemical methods. Several studies have been devoted to the development of biomaterials based on the radiation surface modification process [113–117].

As described earlier, surface chemistry is critical in mediating host and cellular responses. Additionally, functional groups on the surface can be exploited as chemically reactive sites for coupling other function molecules for specific biomedical applications. In this context, gamma irradiation may be useful for the introduction of functional moieties to the material surface. For example, polystyrene (PS) has many attractive features for medical applications but is a typically inert polymer which lacks functional groups. Exposure to gamma radiation yielded carbonyl and ether functional groups on the surface. Carbonyl groups were presented below the top few molecular layers of ester. Unsaturated carbonyl/acid groups formed a higher proportion of the total carbonyls with increasing depth, and the extent of interior oxidation was linear with gamma dosage [118]. In another study, polyethersulfone (PES) was subjected to gamma radiation for the purpose of changing the material's innate hydrophobicity, in order to make render the surface less susceptible to fouling [119]; this process was shown to be remarkable for homogeneity of the modified surface. One of the main challenges in designing blood-contacting biomaterials lies in the need to prevent thrombus formation. Proper tailoring of the biomaterial surface is aimed at reducing the adsorption of clot-initiating proteins and the adhesion of platelets. In one study, polyethylene glycol methacrylate (PEG-MA) with different molecular weights was conjugated on the surface of PE films by gamma irradiation, and results showed less adsorption of proteins and adhesion of platelets on PE film surfaces after modification [120]. The degree of grafting is found to be strongly dependent on the reaction conditions, as well as the storage time and temperature of the irradiated film prior to the reaction. Additionally, reaction temperatures can be controlled to keep segmental mobility low such that the free radicals that are produced during the irradiation remain trapped within the matrix.

6.2.2.4 Hydrolysis- and Aminolysis-Induced Modification

Many polyesters such as poly(lactic-glycolic acid) (PLGA), PET, poly(ester urethane) (PU), PLLA and PCL have been used for wide biomedical applications such as drug delivery and medical devices due to their well controllable degradation rate

and mechanical properties [87, 121–123]. As mentioned earlier, surface modification of these polymers is necessary to improve their biocompatibility. Among those surface modification methods available, wet-chemical methods of hydrolysis and aminolysis are used most frequently due to their (1) simple steps, (2) ease of control (3) and scalability to three-dimensional structures. Through hydrolysis and aminolysis, carboxylic acid and amine groups could be produced on the surface of polymer in a highly controlled manner, with minimal erosion.

Hydrolysis of polyesters can be driven by either acidic or basic conditions [86, 124]. However, under acidic conditions, hydrolysis of esters is achieved via electrophilic attack by hydrogen ions on the carbonyl oxygen which requires very strongly acidic conditions and may target the bulk material, instead of being limited to surface hydrolysis. In contrast, under basic conditions, hydrolysis is achieved by nucleophilic attack by hydroxide ions on the carbonyl carbon, which is surface-oriented and results in less bulk hydrolysis. For example, PCL films subjected to alkaline hydrolysis obtained rapid increase in surface wettability, while the surface topography was less changed at microscales and accompanied by little mass loss [46]. However, alkaline hydrolysis typically results in bulk degradation of the PCL film and accelerated loss of structure. In the same study, hydrolysis was limited to the superficial layer of uniaxially drawn PCL, highlighting the effects of post-processing.

Aminolysis is driven by nucleophilic attack on the carbonyl carbon to generate a positively charged tetrahedral intermediate. Aminolysis may be performed either in basic solutions or in an aprotic, polar solvent. Unlike base hydrolysis, the overall activation energy for the aminolysis is low and even negative in organic solvents, resulting in reduced or inverse dependence of aminolysis on the reaction temperature. It has been previously reported that aminolysis rates typically reach a plateau at pH values just above the pKa of the amine in aqueous solutions [125]. In a study on PCL scaffolds for vascular tissue engineering applications, aminolysis was performed to introduce amino groups through reaction surface of PCL with 1,6-hexanediamine [121]. It was found that there was a direct correlation between the amount of amino groups generated on the PCL film surface and the concentrations of 1,6-hexanediamine concentrations (0–14%). The amount of amino groups also increased with a prolonged exposure duration, reaching a maximum value at 1 h. Incubation beyond that resulted in a decrease in free amine groups, possibly due to auto-polymerisation with terminal carboxyl groups or degradation of the superficial layer. The exposed amino groups could subsequently be used as anchor sites for the conjugation of protein such as gelatin, chitosan and collagen. The follow-up endothelial cell culture proved that the cell attachment and proliferation ratios were obviously improved, and the cells showed a similar morphology to those cultured on tissue culture polystyrene surfaces.

6.3 Techniques for Analysing Modified Surfaces

Surface characterisation of a modified material is an essential step in determining whether the surface modification is successful and whether the modified surface can satisfy the requirements of its intended application [75, 88, 126]. There are various techniques to characterise the surface properties of a material, and they are broadly categorised into physical, chemical and biological techniques based on the nature of the information intended to be elicited out. In general, physical techniques focus on the surface tension, topography and weight variation, and chemical techniques provide information on the chemical structure and chemical composition of the surface, and biological techniques assess the biocompatibility and cellular responses to the surface. The most common techniques used in the characterisation of polymeric biomaterial surfaces for tissue engineering applications are summarised in Table 6.2 and presented in the following sections.

6.3.1 Physical Characterisation

6.3.1.1 Contact Angle Measurement

As most of polymeric biomaterials will apply in an aqueous environment during their applications in vitro or in vivo. Therefore, it is important to study the interaction between water and the surface of the material, also known as material surface wettability. This reactivity of water with material surface is central in molecular

Table 6.2 Most common techniques for characterising polymeric biomaterial surfaces

Technique	Category	Probe	Information	Requirement
Contact angle	Physical	Liquid drop	Surface energy	Clean, homogeneous, non-porous surface
SEM	Physical	Electrons	Surface topography	Vacuum, conductive sample surface
AFM	Physical	Cantilever	Surface topography, composition, roughness	Clean
QCM	Physical	Quartz crystal resonator.	Mass change	Polymer need to be pre-coated on resonator surface
FTIR	Chemical	Infrared light	Surface composition, binding state	Bulk phase having no overlapping IR absorption with surface molecules
XPS	Chemical	X-ray/ electrons	Chemical composition, binding state	Vacuum, separate elemental analysis for hydrogen
TOF-SIMS	Chemical	Ions	Surface composition	Vacuum, samples stored in aluminium foil or clean glass containers

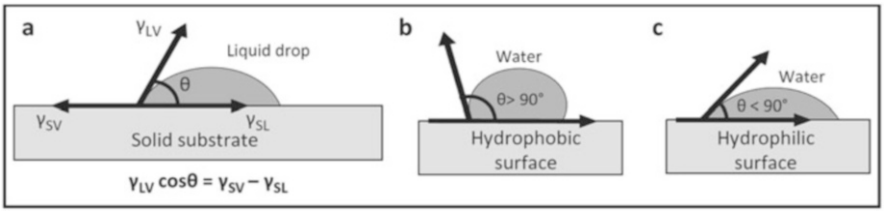


Fig. 6.1 Schematic representation of (**a**) the relation between the contact angle θ and the surface tensions at the three interfaces, (**b**) a drop of water on a hydrophobic surface and (**c**) a drop of water on a hydrophilic surface. (Reprinted from [129], with permission from Springer)

self-association of water at the interface, leading to the formation of water structure that governs the selective adsorption of proteins on the material surface [127]. The wettability of a surface is typically revealed by placing a drop of liquid onto the surface and measuring the contact angle – the angle between the liquid–vapour interface and the solid surface [128]. The contact angle θ is related to the surface tensions at the liquid–vapour, solid–vapour and solid–liquid interfaces (represented by γ_{LV}, γ_{SV} and γ_{SL}) in Young's equation given in Fig. 6.1a [129, 130]. In general, a stronger attraction between the liquid and the surface leads to a lower contact angle. For biomaterials, deionised water is typically used as the probe liquid. Surfaces with a contact angle of more than 90° are generally defined as hydrophobic (Fig. 6.1b), whereas surfaces with a contact angle of less than 90° are generally defined as hydrophilic (Fig. 6.1c). In surface modification experiments, comparing the contact angle value before the modification with the value after the modification can be used to examine the effectiveness of the modification process [1].

Contact angle measurements are typically done on a goniometer, an instrument used for precise measurements of angles. A modern goniometer is equipped with a camera and a software where the researcher can define the solid–liquid interface (also known as drop baseline) and set the fitting method to fit the drop shape. Beside static measurements, dynamic contact angle measurements can also be performed to enhance sensitivity. Dynamic techniques include increasing and decreasing the drop volume and tilting the surface to determine the advancing and receding contact angles. While contact angle measurement is valuable for assessing surface wettability, it is not reliable for heterogeneous surfaces where the wettability differs at various parts of the sample and porous samples where the drop is absorbed into the material [131]. Contact angle measurements also do not offer information on the surface chemistry and topography changes after surface modification. Hence, other characterisation techniques are often performed paralleling with contact angle measurements to provide a full evaluation on the result of a surface modification process [1, 88]. In Fig. 6.2, it shows the contact angle of PEEK is 100.3°. After the sulphonation and hydrothermal treatment, the surface contact angle changes to 70.1° with improved hydrophilicity due to the porous structures on the surface. The contact angle of SPEEK-Sr becomes 25.7°, which is more hydrophilic due to the presence of dopamine on the surface [128].

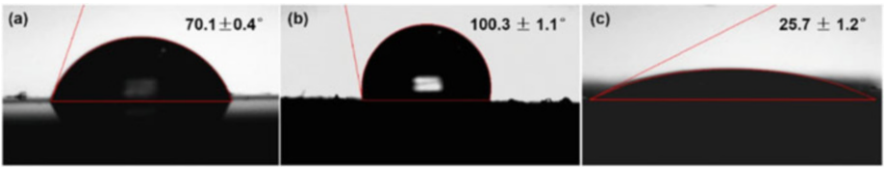


Fig. 6.2 Contact angles of PEEK surface with different treatments. (**a**) PEEK; (**b**) SPEEK-H; (**c**) SPEEK-Sr. (Reprinted from Hu et al. [128] with permission from Springer)

6.3.1.2 Scanning Electron Microscopy (SEM)

Information on the surface morphology and topography of a polymeric biomaterial can be obtained by various microscopic techniques, depending on the dimension of the surface, the desired lateral resolution, the depth of the surface and the sample environment. While optical microscopy is the easiest to use and least invasive among the techniques, its lateral resolution is limited to the wavelength of light, which is around 300 nm. Hence, SEM, which reveals surface features at nanometre lateral resolutions, is often the preferred technique to visualise the surface topography of a biomaterial [132–135]. In SEM, a beam of electrons is directed onto the sample under vacuum, and the resultant interaction between the electrons and the sample surface causes an emission of secondary electrons, which are collected by the detector to produce an electron micrograph. Samples that are not electrically conductive need to be sputter-coated with a thin metallic coating to prevent electrical charging (the accumulation of electrons) on the sample. As the analysis takes place under vacuum to prevent scattering of electrons by air molecules, samples containing cells and biological tissues have to be fixed and dried to ensure that the biological components remain stable in vacuum [133, 136]. Figure 6.3 shows that the cells adhered and spread on the surface of substrates and displayed characteristic star/slayed-shaped morphologies on day 3. The cells reached around 80% confluency on day 7, and the cells formed confluent monolayer sheets for all groups on day 28 [133].

Some samples, such as hydrated polymers and surfaces modified with adsorbed molecules, may not be suitable for SEM due to their instability in vacuum. As the SEM operates in a dry environment, the information obtained may not truly represent the actual surface topography in physiological conditions. Nevertheless, the ease of operation and the ease of interpreting the images make SEM one of the most universal techniques to analyse surface topography of a biomaterial at the nanometre scale. The development of the environmental SEM (ESEM) permits wet, uncoated specimens to be studied; however, beside high cost of equipment, the restricted possible minimum magnification may limit its applications as well [137].

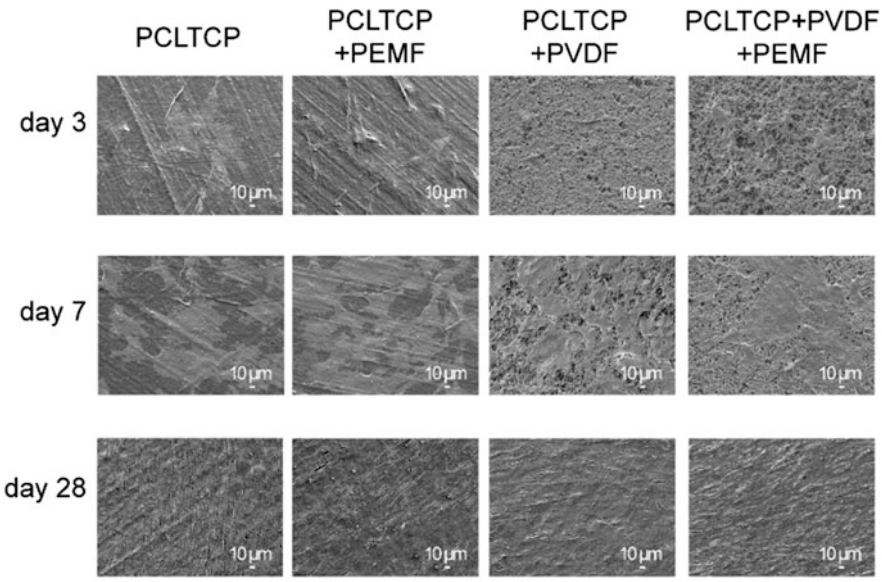


Fig. 6.3 Fixed cell morphology on PCL-TCP without (PCL-TCP) and with pulsed electromagnetic field (PEMF) (PCL-TCP + PEMF) and on PVDF-coated PCL-TCP without (PCL-TCP + PVDF) and with PEMF (PCL-TCP + PVDF+PEMF) observed using SEM on day 3, 7 and 28. (Reprinted from Dong et al. [133], with permission from MDPI)

6.3.1.3 Atomic Force or Scanning Force Microscopy (AFM or SFM)

AFM (SFM) offers three dimensional (3D) and high resolution information at the sample surface and is capable of detecting surface features of several nanometres in depth or height, unlike SEM which offers two dimensional information and limited to tens of nanometres scale only. In fact, AFM can be used to resolve molecules or even single atoms adsorbed on a smooth surface, and its sensitivity allows researchers to obtain images of delicate biological features [130, 138]. An AFM consists of a sharp tip attached to a flexible microscale cantilever (Fig. 6.4). When the tip is scanned across the sample surface, attractive and repulsive forces between the tip and sample cause the cantilever to deflect vertically. The deflection is detected by a photodiode via a laser beam reflected off the top of the cantilever, and the signal is processed into a topographical image. Depending on the scan mode, a constant force or constant height between the tip and the sample is maintained by a feedback loop, which controls the movement of the piezoelectric scanner holding the sample [139].

Depending on the sample's properties and the application, the AFM can be operated in a number of modes. One frequently used mode is the contact mode, where the tip is in constant contact with the sample. While the contact mode offers the highest resolution, the shear forces applied by the tip may damage soft samples and surfaces with weakly adsorbed molecules. Another frequently used mode is the

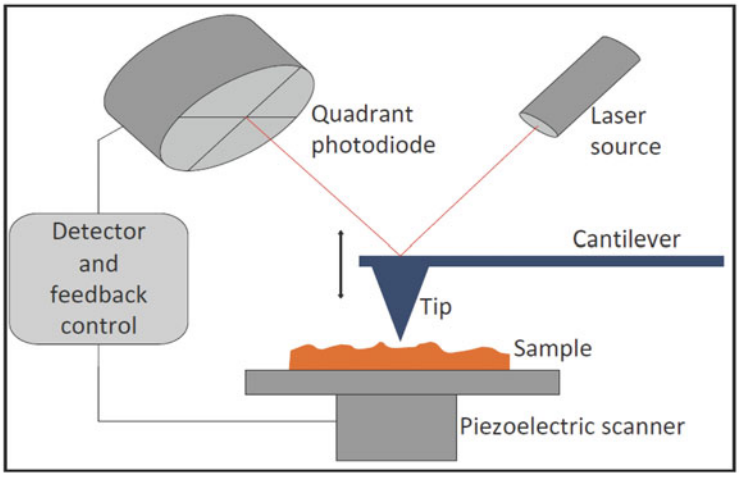


Fig. 6.4 Schematic representation of the working principle of AFM

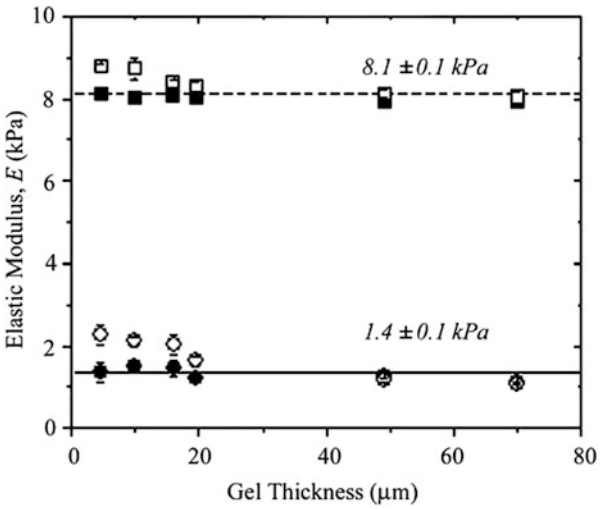


Fig. 6.5 The elasticities of polyacrylamide thin films (5% acrylamide solutions were mixed with either 0.3% (squares) or 0.03% (circles) bis-acrylamide crosslinker and polymerised). (Reprinted from Engler et al. [141], with permission from Elsevier)

tapping mode, where the cantilever is oscillating above the moving sample and the changes in amplitude and phase are tracked. Since the tip is not in contact with the sample, the tapping mode is suitable for soft samples. Besides high resolutions, other advantages of the AFM include its ability to operate in a variety of environments including air and aqueous solutions, its ability to measure interaction forces between a surface and adsorbed molecules and its ability to measure electrical properties (e.g., charge density) of a surface [75]. In addition, the AFM can also be used to obtain the mechanical (modulus, stiffness, viscoelastic, frictional) and magnetic properties [140, 141]. Figure 6.5 shows that the elasticities of polyacrylamide are independent of thickness as measured by AFM indentation.

On the other hand, limiting characteristics of the AFM are a much slower scanning speed, small scanning area (less than 100 μm wide), sample damage or sample movement caused by shear forces from the tip and probe damage caused by hard samples with steep features.

6.3.1.4 Quartz Crystal Microbalance (QCM)

A quartz crystal microbalance (QCM), is also known as quartz microbalance (QMB), or quartz crystal nanobalance (QCN)), which measures a mass variation per unit area through measuring the change in frequency of a quartz crystal resonator. The QCM takes advantage of the piezoelectric effect of the quartz crystal, converts the surface mass change of the quartz crystal into the frequency change of the output electrical signal of the quartz crystal oscillation circuit and then obtains the high-precision data through the computer and other auxiliary equipment [142]. The measurement accuracy can be nanogram level and theoretically can measure the mass change equivalent to a single molecular layer or atomic layer of a fraction. QCM was used to determine the affinity of molecules (proteins, in particular) to surfaces functionalised with recognition sites and interactions between biomolecules [143, 144]. For example, the capture ability of heparin on cellulose nanocrystals surface was evaluated with QCM by measuring changes in resonance frequency shifts (Δf) and energy dissipation (ΔD) as a function of time under constant flows [145]. Larger entities such as bacteria, viruses and polymers are investigated as well [146]. With the rapid development of science and technology, QCM has also been greatly updated, and in combination with other instruments, QCM can also be used in more areas [142, 147, 148]. For example, combined with light microscopes, cells behaviour could be observed on the chip's surface; combined with electrochemical cell chamber, the changes of electrochemical properties could be detected. While the QCM is a direct and sensitive method to characterise surface of material, it should be noted that the adsorption protein obtained using QCM test is a 'wet protein', which includes information about the water molecules associated with the protein. Therefore, the QCM will result in a much greater amount of adsorption protein than that of surface plasmon resonance (SPR) test [149]. Figure 6.6 shows the fibrinogen adsorbed on the surface of SAM-OEG membrane by both QCM and SPR. The adsorption protein obtained from the QCM is 19.5 ng/cm^2, while the adsorption protein obtained from the SPR test is 0.93 ng/cm^2. It can be seen that the adsorption of fibrinogen on the membrane surface measured by QCM is 20 times higher than that obtained by SPR.

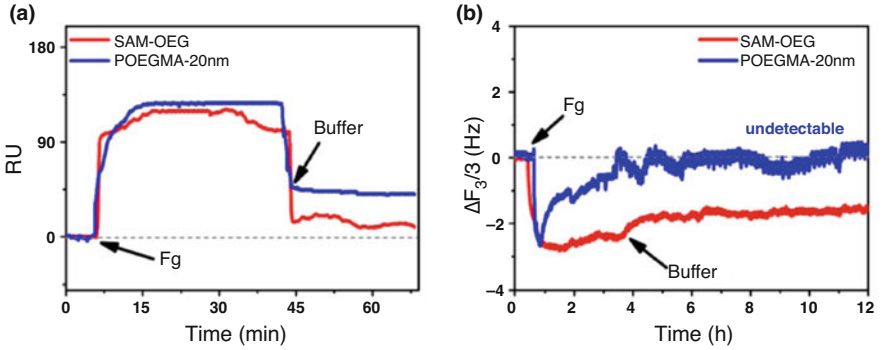


Fig. 6.6 Fibrinogen adsorption (0.1 mg/mL) on SAM-OEG and POEGMA membranes surface measured by (**a**) SPR and (**b**) QCM-D. (Reprinted with permission from Luan et al. [149], with permission from American Chemical Society)

6.3.2 Chemical Characterisation

6.3.2.1 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (FTIR)

The surface chemistry of polymeric biomaterials can be investigated by various spectroscopic techniques, and ATR-FTIR is one spectroscopic technique that is widely used [1, 75, 150]. The principle behind Fourier transform infrared spectroscopy (FTIR) is that various organic functional groups absorb light at specific wavelengths in the infrared (IR) spectrum that are characteristic of their vibrational modes [151]. Hence, FTIR allows quantitative determination of a sample's chemical composition and is a powerful tool to track the chemical changes that occur after a chemical reaction. FTIR is typically performed by passing a beam of IR light through a solid sample blended with a salt transparent to IR or a liquid sample sandwiched between two salt discs. However, since most polymeric biomaterials are opaque to IR light and cannot be homogeneously blended with salt, they are analysed in the attenuated total reflection mode, where the surface of the sample is pressed onto an inorganic crystal (e.g., ZnSe or Ge) and a beam of IR light is directed toward the crystal–sample interface. Despite total reflection at the interface, the incident IR beam penetrates the sample in the form of an evanescent wave. After the sample absorbs light at specific wavelengths, the reflected beam leaves the crystal and is converted to an IR spectrum by the FTIR system [152]. Figure 6.7 shows that two distinct bands were observed at 1560 and 3419 cm^{-1} in P-JAG scaffolds spectrum. The band observed at 1560 cm^{-1} is assigned to the N–H bend of primary amines in JAG peptides and the broad band at 3419 cm^{-1} corresponds to N–H stretch of primary and secondary amines in JAG peptides. Therefore, the spectrum suggested that the Jagged-1 peptides were successfully immobilised onto the surface of scaffolds [75].

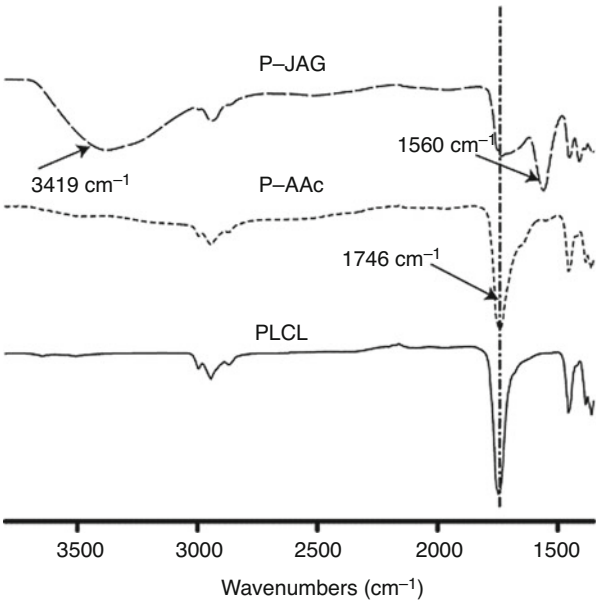
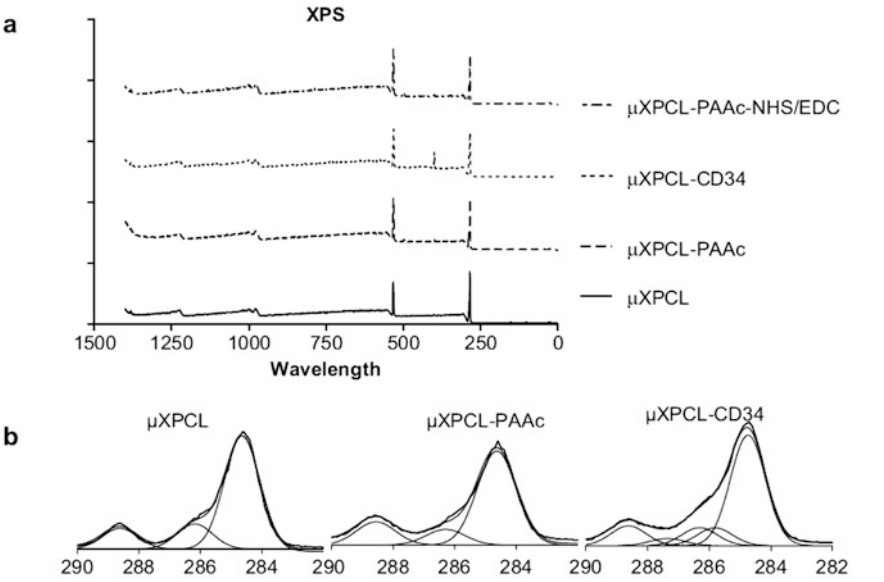


Fig. 6.7 ATR-FTIR spectra of PLCL (continuous line), P-AAc (short-dashed line) and P-JAG (long-dashed line) scaffolds. (Reprinted with permission from Wen et al. [75], with permission from American Chemical Society)

While the ATR-FTIR is fast and easy to use, it is not a very surface-specific technique because the probe depth ranges from several hundred nanometres to several micrometres. In contrast, a layer of immobilised molecules (e.g., proteins) on a surface may only be several to tens of nanometres thick. For polymeric biomaterials, the bulk phase's IR absorption may mask the peaks of the immobilised molecules, making it impossible to detect the presence of the immobilised molecules [126]. Because of this reason, ATR-FTIR is limited to the analysis of homogeneous samples or thin layers of organic molecules on inorganic substrates, where the IR absorptions of the inorganic phase do not overlap with that of the organic molecules.

6.3.2.2 X-Ray Photoelectron Spectroscopy (XPS)

XPS is a more powerful and more surface-specific technique than ATR-FTIR for analysing the surface chemical composition of polymeric biomaterial, with a sampling depth of less than 10 nm [126]. In XPS, X-rays are radiated onto the sample to excite the electrons in the atoms, causing the electrons at the surface of the sample to eject. The quantity of the ejected electrons is measured as a function of the incident energy by the photoelectron spectrometer. As each chemical element has a characteristic spectrum, the overall spectrum can be used to quantitatively determine the elemental composition in the sample surface. Although the incident X-ray can penetrate far into the sample surface, only the electrons within 10 nm of the surface can escape from the sample surface without obstruction [153]. This explains the high surface specificity of XPS. The sampling depth and surface sensitivity can be further



Atomic Ratio (%)					
Binding Energy (eV)	-C-H ~284.6	-C-O ~286.2	-O-C=O ~288.6	-C-N ~285.8	-O-C-N ~287.4
Pristine	71.2	15.8	13.0	-	-
μXPCL-PAAc	70.7	11.8	17.5	-	-
μXPCL-CD34	63.7	10.4	11.5	10.3	4.1

Fig. 6.8 XPS survey of material surfaces. (**a**) XPS can be used to obtain a wide survey spectra of material surfaces. Here, polycaprolactone (*PCL*) films were modified with polyacrylic acid (*pAAc*) to allow conjugation of a protein (CD34 antibody), as identified by the peak at 286.4 eV. (**b**) Relative intensity of the deconvoluted C1S spectra can also be used to show increase in carboxyl groups following PAAc engraftment, followed by introduction of peptide groups following CD34 antibody conjugation. (Reprinted with permission from Chong et al. [88], with permission from Elsevier)

controlled by changing the angle between incident X-ray and the sample surface. As XPS is surface specific, it is a useful tool to detect the presence of immobilised molecules on the surface of a modified biomaterial. For example, protein molecules grafted onto an aliphatic polymer can be detected and quantified by the N1 peaks, since nitrogen atoms are present in the protein molecules but not in the polymer [1, 75, 88] (Fig. 6.8). Besides elemental quantification, chemical state information can also be obtained from XPS, as the chemical environment around an atom can influence the binding energy of the ejected electrons and cause a chemical shift. One example is carbon, which exhibits different binding energy in various functional groups. As a result, the chemical shift of the C1 peak can be used to identify certain functional groups [70, 88]. However, one major limitation of XPS is its inability to

detect hydrogen or helium, which can lead to inaccurate information on the elemental composition of a hydrogen-containing sample. While XPS is sensitive, it requires a vacuum environment to prevent scattering of ejected electrons by gas molecules. This means that, similar to SEM, XPS is only suitable for dry samples and may not be suitable for surfaces with adsorbed molecules. In addition, caution has to be taken for polymers and biomolecules as they can degrade under X-ray radiation, leading to altered chemical properties.

6.3.2.3 Time-of-Flight Secondary Ion Mass Spectroscopy (TOF-SIMS)

TOF-SIMS is a spectroscopy technique for obtaining information on the chemical composition of a solid surface. With a sampling depth of 1–2 nm, it has an even higher surface specificity than XPS. Originally developed for the analysis of inorganic materials, TOF-SIMS has progressed into a versatile tool for the analysis of organic molecules, biomolecules and polymers, and it has an advantage over XPS for being able to identify hydrogen [126, 154]. In TOF-SIMS, a beam of energetic primary ions, usually argon or gallium, bombard onto the sample surface, generating a collision cascade where the primary ions transfer energy to the sample. The collision causes atoms and molecules to sputter from the sample surface. A small portion of the sputtered particles are ionised to produce secondary ions, which are accelerated via an electric field and then made to travel a distance in a field-free drift region before reaching the detector. As the speed of the ions depends on their masses, the time of flight of an ion provides information on its mass and eventually its identity. As each chemical structure has its characteristic mass spectrum, analysis of the final spectrum can provide significant information on the chemical composition of the sample surface [155]. TOF-SIMS can be operated in two modes – static and dynamic. Static TOF-SIMS scans the sample surface with a low-energy primary ion beam to produce a static analysis of the topmost layer of the sample. Dynamic TOF-SIMS uses a high-energy primary ion beam to erode the sample surface continuously and record the real-time signal simultaneously, producing a depth profile of the chemical compositions layer by layer into the bulk. While TOF-SIMS is highly sensitive for surface analysis, it can also be sensitive to contamination. Common contaminants include plasticisers found in plastic containers and silicones found in double-sided tape used to secure samples [154]. Because of this reason, TOF-SIMS samples should be stored in aluminium foil or clean glass containers. Like SEM and XPS, TOF-SIMS is performed in a high-vacuum environment, so samples should be dry and stable in vacuum. Another limitation of TOF-SIMS is the huge quantity of data generated, as every pixel of a two-dimensional image contains a full mass spectrum. Analysis of the data can be extremely time-consuming and complicated if one does not know the sample well. To simplify data analysis and maximise the amount of meaningful information, computational multivariate analysis methods are developed to process TOF-SIMS images [156].

6.4 Characterisation of Biocompatibility

Having discussed the physical and chemical characterisation methods, this section focuses on the biological characterisation of surface-modified biomaterials. In general, the ISO 10993 provides an extensive set of guidelines to evaluate the safety and efficacy of devices and may be applied toward material testing. It also provides an opportunity for the investigator to better understand the safety profiles of the material used, which may be helpful in guiding the rational selection of materials for design of medical products. Many of these tests are contextual and need to be appropriately selected for use in specific applications. The ISO 10993-4, for example, deals with material interactions with blood and provides the basis for selection and design of appropriate tests for blood-contacting surfaces, such as engineered blood vessels. Similarly, the ISO 10993-5 provides basic guidelines for cytocompatibility testing. At this point, it should be noted, for eventual translation into the market, that the European Committee (EC) adopts this standard and makes it part of their technical review in regulatory evaluation of safety of medical devices.

6.4.1 A Note on the Use of the ISO 109993

In continuing this discussion, it is important to stress that any given material cannot be declared to be 'universally biocompatible'; biocompatibility can only defined only in the context of application. Correspondingly, regulatory bodies such as the US FDA are unable to provide a blanket approval for any group or type of polymers for medical applications. Instead, the selection and use of material for a device, along with appropriate tests to demonstrate safety for use in the identified medical application will need to be carefully designed. For regulatory submissions, the control and documentation of this process is covered as part of a quality system, in accordance with good manufacturing practices. Discussion of design controls is beyond the scope of this chapter and readers are instead directed to Section 4 of the ISO 13485, as well as the US FDA guidance document on design controls. In the subsequent sections, the following will be discussed:

ISO 109993-1: Evaluation and testing within a risk management process Part 1 provides background and overview on the approach toward biocompatibility testing.

ISO 10993-4: Selection of Tests for Interactions with Blood, ISO 10993-5: Tests for in vitro Cytotoxicity Discussion of Part 4 and Part 5 will provide examples of the development and use of assays for the evaluation of specific aspects of biocompatibility.

6.4.2 ISO 10993-1: Evaluation and Testing Within a Risk Management Process

The ISO 10993-1 is an important document as it provides a starting point toward developing a plan for the evaluation of biological responses to a medical device. It includes a description of the general principles applying to biological evaluation of medical devices, followed by a framework to categorise medical devices according to the nature and duration of contact with the body. Accordingly, appropriate data sets and / or relevant parts of the ISO 10993 may be selected to guide testing, in order to address identified gaps in knowledge about the device and material being used.

The general principles provide useful insight into the thought process behind the risk-based approach to biological evaluation. A critical consideration is that testing should only be performed where there is insufficient information to perform an adequate risk assessment (the process of doing so may be accessed in the ISO 10993-2). A flow chart is also provided in this section to guide users through considerations in categorising a given device, including whether the patient contact is involved, formulation used, manufacturing processes, geometry and physical properties, as well as the nature of use.

Subsequently, the medical device may be categorised as a surface medical device, externally communicating device or implant medical device, and further sub-categorised on the type of tissue contact. Following further consideration of the contact duration (limited, prolonged or long-term), relevant endpoints of biological evaluation are identified, that can indicate the data sets needed to assess biological safety. Following data gap analysis, a list of required data sets may then be identified, which can be generated in conformance with appropriate standards. For example, a scaffold being used in vascular tissue engineering, may be seen as an implant medical device that comes in contact with blood, with long term duration of more than 30 days. Accordingly, the typical biological evaluation endpoints will typically include Haemocompatibility and In Vitro Cytotoxicity (discussed later).

6.4.3 ISO 10993-4: Selection of Tests for Interactions with Blood

The ISO 10993-4 deals with studying interactions with blood. It includes a framework to categorise blood-contacting materials (based on intended use and duration of contact), an overview of governing principles in studying interactions with blood and selected tests, including the rationale behind use of data generated from such tests.

6.4.3.1 Categorisation of Device Types

Blood contacting devices, in the context of this document, do not include devices in which the contacted blood does not return to or reside in the body. Accordingly, blood contacting devices may be broadly represented as external communicating devices or implants (such as heart valves. External communicating devices may be further categorised as devices that serve as an indirect blood path (such as blood collection devices) or those that directly contact circulating blood (such as atherectomy devices). It should be noted that the context of application impacts the level of risk significantly – intravascular catheters may be used for a variety of applications and may be categorised as indirectly or directly contacting circulating blood, depending on the use. It is thus the intended use or site of application that dictates the level of risks, rather than the material used or surface modifications employed.

6.4.3.2 Characterisation of Blood Interactions

Similar to ISO 10993-1, a decision tree can be used to decide whether testing for interactions with blood should be performed. Additionally, a table is provided to identify the category of tests for consideration; these include haemolysis (material-induced or mechanically induced) and thrombosis (coagulation, platelet activation, complement, haematology and ex vivo/in vivo. While not prescriptive nature, the standard provides rationale for the selection of these tests and essential considerations in the design of such tests. Some recommended tests include PTT assays and thrombosis and are performed in the context of the device (discussed later).

6.4.3.3 Types of Tests

The recommended tests in 10993-5 are divided into categories based on the primary process being measured. Taking the example of a vascular graft, in order to evaluate thrombosis in vivo, common measures include percentage occlusion of the graft following a period of implantation. The standards do not dictate testing protocols due to the diverse nature of devices being evaluated and that blood interactions tend to be context-specific. It is also not possible to include target numbers that qualify a given material to be 'haemocompatible'. Rather, a set of principles are provided, suggesting the rationalisation necessary to justify the choice and design of tests. Some examples are discussed as follows:

In Vitro Tests In vitro testing typically involves exposure of the material to blood and may include bench tops models (such as the Chandler loop test model [157]) that simulate physiological conditions, particularly that of an 'anticipated worst case scenario'. In such a set-up, materials to be tested are exposed to collected blood and the interactions with blood are studied through the use of assays in bench top

settings. Partial Thromboplastin Time (PTT) is one such assay in which the time taken to induce clotting of recalcified citrated plasma is measured, following the addition of partial thromboplastin. To perform coagulation testing of medical devices and materials, exposure to the device or material serves to activate the coagulation. By measuring the change in PTT following exposure, a relative measure of surface coagulability may be established for a range of modified surfaces. The PTT and other in vitro tests provide repeatable settings, avoid the use of animals and also provide a cost-effective way to perform initial evaluations. They are, however, limited in their ability to replicate physiological settings and longer-term studies typically involved the use of animals. For more detailed discussion on blood compatibility testing, the following review provides further elaboration of testing methodology [158].

Ex Vivo Tests Ex vivo testing involves directing circulating blood to contact with a test material. These include 'open' systems such as the Dudley clotting test, where a tube is inserted into a test subject via a catheter and the time taken for blood to stop dripping from the open end of the tube can be compared. Popular 'closed' systems include AV shunts, where the test material is directly exposed to circulating blood. Typically, tests involving AV shunts may be continued for extended durations and more information may be derived. For example, retrieved shunts may be examined for presence of thrombi on the surface, or changes in patency may be measured to indicate thrombogenic responses. Luminal surfaces may also be studied for platelet adhesion, while downstream vasculature may be monitored for embolic events.

In Vivo Tests In vivo testing of devices typically involves implantation of a device in the intended site of use, to more closely mimic clinical application. These typically require greater planning and can provide more data temporally and across a range of diverse tests. For instance, serial monitoring of implanted vascular grafts by arteriograms provide vital information on the development of thrombi and changes in patency over time. In testing of modified surfaces (not necessarily in the specific context of a device), the non-anticoagulated venous implant (NAVI) or anticoagulated venous implant (AVI) models are used. These involve inserting of device materials formed into catheter shapes into the veins of animals for up to 4 h, followed by gross assessment of amount of thrombus on the material/catheter surface. The technique faces several shortcomings, including limited time exposures, variability of results and operator dependence. Perhaps more critically, the high flow environments lead to very low levels of surface-associated thrombus. Particularly in AVI settings, this results in most material surfaces being labelled non-thrombogenic (even if it is not the case).

6.4.4 ISO 10993-5: Tests for In Vitro Cytotoxicity

The ISO 10993-5 deals primarily with the evaluation of cytotoxicity or, more accurately in this case, the lack thereof. Broadly, it involves exposing test cells to

Table 6.3 Summary of cytotoxicity tests and their corresponding cell lines

Test name	Suitable cell lines
Neutral red uptake	BALB/c 3T3 cells, clone 31 CRB 9005
Colony-forming cytotoxicity	V79
MTT cytotoxicity	L-929
XTT cytotoxicity	L-929

Referenced from ISO 10993-5:2009, Annexes A–D. Copyright remains with ISO

the material, either directly or indirectly, followed by evaluation of viability. Some tests available for such evaluations are listed in Table 6.3.

In general, a numerical grade of >2 in qualitative scoring is considered cytotoxic. For qualitative testing, reduction of cell viability by $>30\%$ shall indicate cytotoxic effects. Under these conditions, significant considerations shall be given to the surface modification procedure, which could include but is not limited to (1) use of cross-linking agents, (2) chemical alteration of biomaterial molecular structure and (3) surface-modified coating chemistry and its cytotoxicity effects.

At this point, it is critical to highlight that the evaluation of cytocompatibility alone is not a direct measure of surface modification efficacy (except where the modification is performed specifically to improve cytocompatibility). It does, however, provide important indications on the safety profile of the surface coating process, in order to flag out unexpected cytotoxic events arising from the modification process.

6.4.4.1 Direct Contact

Direct contact methods involve the direct exposure of cells to the material surface, followed by evaluation of cell viability. One important test requirement would be to ensure that there is at least one flat surface (no specific requirement on roughness is provided). In addition, if the biomaterial is meant to be used sterile, then it shall be sterilised accordingly before testing is done. Otherwise, the basic principles of aseptic handling during testing shall apply. The selection of cell lines to be used for testing shall, in principle, follows the requirements of the standard. However, concession is also given to situations where a specific response to a selected cell line is desired; in this case, cell line reproducibility and accuracy of response need to be demonstrated. Testing the material involves culturing the cells to subconfluency on standard plates and subsequently placing the material directly on the cells. Cell viability is then tracked over multiple days and may also be morphologically observed under microscope. Important test requirements are that appropriate experimental controls (both positive and negative) should be included and that the biomaterial shall only cover one-tenth of the exposed surface area of the cell layer.

Determination of cytotoxicity may be performed using qualitative and quantitative measurements although it is preferred that quantitative measurements are taken.

Table 6.4 Reactivity grades for direct contact test

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 10 cm
4	Severe	Zone extending farther than 10 cm beyond specimen

Adapted from ISO 10993-5:2009, Section 8.5. Copyright remains with ISO

If qualitative measurements are taken, the following table (Table 6.4) provides the guidelines to which cytotoxicity shall be measured.

6.4.4.2 Exposure to Liquid Extracts

This process involves the incubation of the modified material in an extraction fluid medium, to which the cells are subsequently exposed. Extraction conditions generally follow the principle of simulating or exaggerating clinical use conditions without causing significant changes in the biomaterial. For this purpose, the extraction vehicle can be culture medium, physiological saline or any other justifiable medium. Importantly, to fulfil the requirements of the standard, the extraction vehicle(s) should allow extraction of polar and non-polar elements.

The extraction conditions shall be conducted without causing significant changes in the biomaterial and therefore should be chosen carefully. Generally, normal cell culture condition of 37 °C for a period of 24 ± 2 h is applied. However, raised temperatures and durations of extraction may be applied provided that the chemistry of the biomaterial is unaffected, and the intended use of the biomaterial justifies the extraction conditions. Additionally, in situations where the cumulative contact of the biomaterial is less than 4 h and is in contact with intact skin or mucosa surfaces, the extraction times shall be at least 4 h.

6.4.4.3 Indirect Contact

Indirect methods are concerned with measuring the leachables from a material. Two methods are most commonly performed. In the agar diffusion method, selected cell lines are grown to subconfluency, and 0.5–2 mass per cent of melted agar is casted over the cells, with a fresh culture medium change. The sample is then placed on top of the agar, followed by a predefined period of incubation (24–74 h) before evaluation. In the filter diffusion method, a surfactant-free filter of pore size 0.45 μm is used. Briefly, an aliquot of a continuously stirred cell suspension is added onto the surfactant-free filter and incubated until subconfluency is achieved. The filters are then transferred onto a layer of solidified agar (cell side facing down) before the biomaterial is placed onto the acellular side of the filter (top side).

6.5 Conclusion

In this chapter, the modification of tissue engineering scaffold surfaces was discussed. Surface modification seeks to confer desirable surface properties, while retaining bulk properties. In tissue engineering scaffolds, this is often difficult to achieve due to the susceptibility of degradable scaffolds to bulk alterations. This has led to innovative approaches that range from physical to chemical approaches, yielding modified surfaces with varied physical, chemical and biological properties. To characterise these changes, various assays have been developed that can help develop a deeper understanding of the effect of surface modification. Particularly for implant applications, the characterisation of biological responses is important toward establishing the safety profile of modified surfaces; these were discussed using the ISO 10993 standards to illustrate how the selection and design of testing methodology is as important as the choice of surface modification techniques in the development of tissue engineering scaffolds.

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