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Surface modification and property analysis of biomedical polymers used for tissue engineering

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Abstract

The response of host organism in macroscopic, cellular and protein levels to biomaterials is, in most cases, closely associated with the materials’ surface properties. In tissue engineering, regenerative medicine and many other biomedical fields, surface engineering of the bio-inert synthetic polymers is often required to introduce bioactive species that can promote cell adhesion, proliferation, viability and enhanced ECM-secretion functions. Up to present, a large number of surface engineering techniques for improving biocompatibility have been well established, the work of which generally contains three main steps: (1) surface modification of the polymeric materials; (2) chemical and physical characterizations; and (3) biocompatibility assessment through cell culture. This review focuses on the principles and practices of surface engineering of biomedical polymers with regards to particular aspects depending on the authors’ research background and opinions. The review starts with an introduction of principles in designing polymeric biomaterial surfaces, followed by introduction of surface modification techniques to improve hydrophilicity, to introduce reactive functional groups and to immobilize functional protein molecules. The chemical and physical characterizations of the modified biomaterials are then discussed with emphasis on several important issues such as surface functional group density, functional layer thickness, protein surface density and bioactivity. Three most commonly used surface composition characterization techniques, i.e. ATR-FTIR, XPS, SIMS, are compared in terms of their penetration depth. Ellipsometry, CD, EPR, SPR and QCM’s principles and applications in analyzing surface proteins are introduced. Finally discussed are frequently applied methods and their principles to evaluate biocompatibility of biomaterials via cell culture. In this section, current techniques and their developments to measure cell adhesion, proliferation, morphology, viability, migration and gene expression are reviewed.

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Keywords: Biomaterials; Surface modification; Biocompatibility; Tissue engineering; Cells

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1. Introduction

Biomaterials for tissue engineering and regenerative medicine should be both biodegradable and bioactive [1]. Synthetic biodegradable polymers such as polylactide (PLA) and poly(lactide-co-glycolide) (PLGA), however, do not possess the necessary specific bioactive abilities to accelerate ECM secretion and regeneration of cultured cells. Considerable efforts have thus been focused on surface engineering to endow the polymer surfaces with abilities to promote cell adhesion, proliferation, and to maintain cell normal phenotype and functions. Correspondingly, clear characterization of the chemical compositions and physical structures of the biomaterial surface has profound scientific importance, leading to insight understandings of cell–biomaterial interactions. On the other hand, to evaluate whether the surface engineered biomaterials can induce desirable cell interactions, in vitro cell culture has to be performed because no universal basic rules are applicable to predict cell behaviors just by knowing certain material surface properties. The most frequently obtained data by cell culture are cell morphology, adhesion and proliferation, etc. More importantly, cell phenotype and functions relevant to the tissues to be regenerated should be elucidated either in protein or gene level, using various molecular biology techniques.

Due to the broad scope and fast development of the biomaterial surface science, dozens of review articles have been contributed to this topic with regards to variable aspects due to the authors’ different backgrounds. Here, just to name a few among the most prominent ones. In a review of 1994 by Ikada [2], it was pointed out that material’s surface has the most critical influence on the biocompatibility and surface modification, is in many cases essential for a conventional polymer to be used as a biomedical material. The review article focused on grafting techniques with the purposes of obtaining lubricious, blood compatible or physiologically bioactive polymer surfaces. In 1996, Elbert and Hubbell [3] explained the interaction mechanism between cells and biomaterial surfaces. Principles of protein adsorption on the biomaterial surfaces and the biological recognition between the cells and adsorbed proteins were described in detail. Further, polymer surface modification approaches to prepare very hydrophilic or superhydrophobic biomaterial surfaces mainly for preparation of blood compatible materials were reviewed. In 1998, from point of view of a surface chemist, Vogler [4] interpreted biomaterial’s surface hydrophilicity/hydrophobicity’s influence on biocompatibility from a perspective of water molecular structure close to the surfaces. In a review of 2002, Kasemo [5] introduced the interactions between biomaterial surfaces and water molecules, protein molecules and cells and tissues. It was also pointed out that bio-recognition is the mechanism behind cell–biomaterial surface interactions. Several application fields including medical implant, biosensor and biochips, tissue engineering, bioelectronics and biomimetic materials were discussed to illustrate that bio-recognition principle is a central concern in an attempt to make a sophisticated, functional surface for specific biointeractions. In 2005, Stevens and Gorge [6] reviewed current approaches to engineer nano-scaled materials surfaces to mimic the nano-scaled patterns of chemical and topographical clues of natural ECM to tailor cell behaviors. More recently (2006), Morra and Cassinelli [7] reviewed biomaterial characterization and modification approaches in the areas of biochemical modification of titanium bone-contacting device, drug eluting stents carrier surfaces and surface immobilization of bioactive polysaccharides. In addition to general reviews, many other reviews are written from particular perspectives. For example, Hersel et al. [8] reviewed RGD modified polymer surfaces, provided information about technical aspects of RGD immobilization on polymers and discussed RGD peptide surface density, spatial arrangement as well as integrin affinity and selectivity on cell responses. Kurella and Dahotre [9] contributed an in-depth discussion of laser surface engineering in surface modification for bio-implants. Morra [10] reviewed surface engineering of biomaterials using hyaluronan. Special surface modification techniques for biomaterials like vapor based polymer coating [11], plasma [12], plasma induced micropatterning [13], plasma and photons [14], DLC coating [15], ion sputtering [16], plasma-based ion-implantation [17], etc. have been reviewed. Specific techniques for biosurface analysis like TOF-SIMS [18,19], ellipsometry [20], surface MALDI mass spectroscopy[21], XPS[22], etc. have also been discussed.

Based on our experiences and recent results in recent years, in this contribution we briefly review the surface engineering, surface characterization and biocompatibility evaluation of polymeric materials for tissue engineering. It is notable that biomaterial surface science is a very broad area under an explosion of techniques and approaches, and full of controversies. Thus, it is unmanageable to give a complete review in a singular paper. This review is by no means comprehensive and inclusive of all previous works, but rather only represents our particular views and understandings. The references and the techniques quoted are just a fraction of those actually building up this area. The aim of this paper is to provide an introductory reading material of the existing and quickly developing background of the ana-
lytical and synthetic polymeric biomaterial surface science to help the readers enter this area quickly.

2. Surface engineering of polymeric biomaterials

2.1. Principles

It has been well known that the interactions between cells and their environments are mediated by the “bio-recognition processes”, the specific binding of the receptors on cell surface with their corresponding ligands [3]. In native tissues in vivo, cells attached on the extracellular matrix (ECM) are mediated by the binding between integrins (receptors on cell surfaces) and ECM adhesion proteins such as fibronectin, vitronectin, laminin and collagen. In addition to the function of a physical support, native ECM also adjusts cell behaviors by presenting various kinds of growth factors to the cells. On the biomaterial surfaces in vitro, the same mechanisms also apply. When foreign materials come into contact with body fluid or cell culture medium, the initial response is protein adsorption atop the materials’ surfaces. Thus, the materials interact with the cells through the adsorbed protein layer. The composition and structure of this protein layer play critical roles in determining subsequent cell behaviors [3].

Based on the understanding of the dominance of the bio-recognition process on cell behaviors, two main strategies in surface engineering of biomaterials are often employed. Firstly, the material surface properties such as chemical composition, hydrophilicity/hydrophobicity, surface charge and roughness, etc. are modulated to a state that the adsorbed proteins can maintain their normal bioactivities. This method, however, cannot induce specific cell behaviors due to the nonspecific protein absorption. The second strategy is to directly immobilize certain biomolecules on the biomaterial surfaces to induce specific cellular responding.

2.2. Improvement of hydrophilicity

Hydrophilicity/hydrophobicity may be the initial parameter affecting protein adsorption. It is well known that hydrophobic surfaces favor the adsorption of proteins from aqueous solution thermodynamically, but may induce strongly irreversible adsorption and denature the protein’s native conformation and bioactivity (a natural conformation of a protein is a prerequisite for its bioactivity). On the other hand, a highly hydrophilic surface may expel any protein molecules and inhibit protein adsorption. It was proposed by Vogler [4] that material’s hydrophilicity/hydrophobicity can be defined using an important parameter, pure water adhesion tension (τ'). Surfaces with τ' > 30 dyn/cm were defined as hydrophilic and those with τ' < 30 dyn/cm hydrophobic. Molecular self-association theory suggests different water structures in the interphase between the biomaterials surface and bulk aqueous phase, i.e. a relatively less-dense water region against hydrophobic surfaces with an open hydrogen-bonded net work and a relatively more dense water region against hydrophilic surfaces with a collapsed hydrogen-bonded net work. Molecular self-association theory further suggests that hydrophobic materials support protein adsorption in an energetically favorable way while hydrophilic materials repel proteins.

Although hydrophilicity/hydrophobicity has been well known with respect to influencing the protein adsorption, its effect on cell behavior has been quite controversial and inconsistent in experimental data. Based on experimental data from many sources, Vogel [4] summarized that the hydrophobic materials with τ' < 30 dyn/cm have good cell attachment while hydrophilic materials do not support cell attachment. However, many other works demonstrated that improved surface hydrophilicity is necessary for hydrophobic materials to support cell adherence and in particular growth. It is now been well accepted in biomaterial community that both very hydrophilic and hydrophobic surface are not good for cell attachment, rather, surfaces with moderate wettability are able to adsorb a proper amount of proteins, and at the same time preserve their natural conformation, resulting in positive cell response [23–28].

Because most synthetic biodegradable polymers are hydrophobic, extensive efforts have thus been devoted towards increasing the biomaterial’s hydrophilicity. One convenient measure is plasma treatment, which can easily introduce polarized groups such as hydroxyl, carboxyl, amino and sulfate groups on polymer surfaces using different reaction gases such as air, NH₃, SO₂, CO₂ or other organic compounds. Another widely used method is to graft hydrophilic polymers on biomaterial’s surfaces through grafting copolymerization of hydrophilic polymers (Fig. 1). The introduction of initiators (radicals or peroxide groups) on the chemically inert surfaces can be realized by Ar₂ plasma treatment, ozone oxidation, γ-ray, electron beam and laser treatment [29–32]. Cerium (IV) induced grafting polymerization is worthy of notation in that it is a pure wet chemistry method and does not need any special equipment like irradiation source and plasma or ozone generator. The reaction mechanism is \(-\text{CH}_2\text{OH} + \text{Ce}^{4+} = -\text{C}^\#\text{HOH} + \text{H}^+ + \text{Ce}^{3+}\), where the asterisk stands for a carbon free radical. A limitation of the cerium (IV) induced grafting polymerization is that it requires polymers possessing alcoholic hydroxyl groups, such as polyvinyl alcohol [33] and cellulose [34]. However, it has been reported that the highly oxidative Ce(IV) can also attack organic substrates without hydroxyl groups to undergo the electron transfer reaction to give radicals [35,36].

We have developed a “photo-oxidization” method, by which peroxide groups can be universally introduced onto polymer surfaces [37–39]. The reaction proceeds by immersing the materials in hydrogen peroxide solution under UV irradiation. Compared with other method, the “photo-oxidization” does not need special instrument and can be performed facilely. A phenomenon observed for both polyurethane (PU) and PLLA was that the amount of the peroxide groups increased with the photo-oxidization time initially and then decreased (Fig. 2). The explanation is that the UV light can induce both the generation and the decomposition of the peroxides, while irradiation for a longer time favors the latter. The peroxide groups can then be used to initiate the grafting polymerization under UV irradiation [37,38] or Fe^{2+} [39]. Hydrophilic polymers such as poly(hydroxyethyl methacrylate) (PHEMA) and poly(acrylamide) have been grafted on polycaprolactone.
(PCL) \[40,41\] and PLA \[42\] surfaces. PU and PCL grafted with PHEMA, poly(methacrylic acid) (PMAA), poly(acrylamide) or poly(N,N-dimethylaminoethyl methacrylate) showed improved hydrophilicity and endothelial cell adhesion \[43–50\]. Moreover, the photo-oxidization strategy allows also mediation of the surface hydrophilicity and the density of the hydrophilic groups by the reaction conditions such as photo-oxidation time (Fig. 3) and grafting time (Fig. 4), which in turn can mediate the adhesion and morphology of endothelial cells \[40,41\]. Figs. 3 and 4 reveal that prolongation of the oxidation time and grafting time can both produce more hydrophilic surfaces brought by the larger density of –COOH groups on PCL surface. With regard to the cell attachment and proliferation, the best cell performance was achieved when the surface contact angle of the PCL-g-PMAA was 70° (the water contact angles quoted in this review are all static sessile drop contact angle).

As mentioned earlier, the concept that moderately hydrophilic material surface induce “good” cell interaction has been widely accepted. However, this is just a very rough idea requiring further detailed studies. Few works have been

**Fig. 1. General scheme of grafting copolymerization on bio-inert polymer surfaces.**

**Fig. 2. Effect of photooxidation time on the content of hydroperoxide on the PLLA surface.**

**Fig. 3. The water contact angle (a) and water absorbance (b) of the PMAA grafted PCL membrane as a function of photo-oxidation time. The grafting copolymerization was conducted in 4 wt% MAA solution at 30 °C for 1 h after photo-oxidized.**

**Fig. 4. The water contact angle (a) and COOH density (b) on PMAA grafted PCL membrane as a function of grafting time. The PCL membrane was photo-oxidized for 6 h at 30 °C before the grafting copolymerization in 4 wt% MAA solution.**
contributed to detailed understanding of the optimal hydrophilicity/hydrophilicity equilibrium for a specific cell’s behavior on a specific material. Our works in recent years found that different optimal surface hydrophilicity is a variable value depending on cell types and specific material surfaces. For example, it was found the best water contact angle for endothelial cell attachment and proliferation is 70° on the PCL-g-PMAA [40,41], while for chondrocytes is 76° (–COOH density, $3.8 \times 10^{-7}$ mol/cm²) on the PLLA-g-PMAA prepared by the same grafting method [51]. When the water contact angle was decreased to 65° (–COOH density, $12.6 \times 10^{-7}$ mol/cm²), the PLLA-g-PMAA exhibited very poor chondrocyte spreading and attachment [51]. Even for a same substrate, the grafting copolymerization methods affect the optimal water contact angle for cell attachment. For example, when Fe²⁺ was used to initiate the grafting polymerization after the photo-oxidization of the PLLA surface, the PLLA-g-PMAA surface showed best chondrocyte attachment and spreading when the water contact angle was 52° (–COOH density, $1.8 \times 10^{-7}$ mol/cm²) [51], which is much lower than 76° (–COOH density, $3.8 \times 10^{-7}$ mol/cm²), the optimal value for the same PLLA-g-PMAA surface prepared by the UV induced grafting copolymerization [51]. The reason for this difference is yet to be studied. One possible reason is that the PMAA chains grafted on the PLLA surface are shorter, more uniform and of higher density when Fe²⁺ is used to initiate the grafting copolymerization, compared with the relatively longer and sparser PMAA chains when UV is used for the initiation. The different hydrophilic PMAA chain densities and chain length may have significant influence not only on the water contact angle, but also, more importantly, on the adhesion protein molecules’ conformation which will directly affect the cell attachment. In conclusion, the “moderate hydrophilicity principle” by no means gives a universally applicable optimal hydrophilicity for cell behaviors, which must differ depending on cell types and specific material surfaces.

It has to note that the cell–material interactions are not only governed by the hydrophilic character of material surfaces even though moderately hydrophilic surfaces have been found to promote cell adhesion best. Other surface properties such as roughness [32] and surface charge [53] also have influences on cell behaviors. Further, biomaterials implanted in human body will interact with body fluids, tissues and cells simultaneously, of which the situation of protein adsorption and cell interaction with the biomaterial surface will be much more complicated than that happens in cell culture mediums in vitro experiments. Thus, the nonspecific relations between cell behaviors and biomaterials’ surface hydrophilicity found in vitro experiments can not be directly assumed applicable in vivo.

2.3. Protein immobilization

Two main classes of proteins have been immobilized on biomaterial surfaces. The first class is adhesive proteins derived from ECM such as fibronectin, laminin, vitronectin and collagen, etc. These proteins can promote cell adhesion and enhance cell attachment via ligand–acceptor interaction. Adhesive peptides containing the Arg–Gly–Asp (RGD) sequence have especially been of much attention [54,55]. This sequence is frequently found in numerous adhesive proteins and is believed as the binding domain to the cell integrins. The second class of proteins are growth factors. Immobilized growth factors are able to modulate cell behaviors such as proliferation and differentiation. Epidermal growth factor (EGF) has been coupled to a polystyrene plate to induce phosphorylation of the EGF receptor [56]. Immobilized EGF could stimulate the DNA synthesis in hepatocytes, as effective as its soluble form [57], while immobilized insulin can stimulate cell growth more effectively than its soluble form [58]. Bone morphogenetic protein (BMP) has been immobilized on biomaterial surface to induce differentiation of the bone cells [59].

To covalently immobilize protein molecules on the chemically inert polymeric biomaterials, reactive groups such as hydroxyl, carboxyl and amino groups must be firstly introduced as coupling sites. A frequently used approach is to graft poly(acrylic acid) or poly(methacrylic acid) to yield carboxyl groups, followed by the activation of the carboxyl groups with water soluble carbodiimide, and finally by the reaction between the activated carboxyl groups and the amino groups of the target proteins [29,40,60–63]. For example, after photo-grafting PMAA onto polymer (in particular polyesters (PEs) such as PLLA and PCL) surfaces, protein molecules, exemplified with collagen in Fig. 5 (lower part), can be covalently immobilized onto the polymer surface via the EDAC chemistry [62,64].

For polyesters hydrolysis and aminolysis can be used to produce the reactive groups. Yamaoka et al. directly grafted gelatin on PLA surface in gelatin/basic solution [65]. An aminolysis method was developed in our lab by simply treating ester-containing polymers such as PCL, PLA and PU in diamino compounds (e.g. diaminohexane) solution, followed by grafting of proteins using glutaraldehyde as coupling reagent (Fig. 5, upper part) [66–69]. One problem of the hydrolysis/aminolysis method is the molecular weight of the biodegradable materials will be partially sacrificed, thus the reaction conditions should be well controlled.

One concern of the covalently protein immobilization is that the natural conformation of the grafted proteins might be changed. In fact, the protein molecules do not necessarily have to

![Fig. 5. Schematic representations of methods to immobilize biomacromolecules on polyester surface.](image-url)
be covalently bound, but instead can be immobilized physically. However, simply casting a protein solution on a hydrophobic surface can only yield an uneven and non-stable protein layer, and in some cases cannot form any protein layer at all. To obtain a stable protein layer, special treatments of the material surface should be performed in prior. Yang et al. [70] obtained a stable collagen layer on PLA surface by treating PLA with ammonia plasma to increase hydrophilicity, followed by collagen coating. Bissona et al. [71] grafted poly(acrylic acid) on poly(ethylene terephthalate) (PET) film to produce a negatively charged surface, and then coated collagen via electrostatic interaction between the negatively charged PET surface and the positively charged collagen molecules. A technique called “entrapment” is to swell the polymer surface using a mixture of a solvent and a non-solvent containing target biomacromolecules, then the biomacromolecules were “entrapped” on material surface by immersing the material into the pure non-solvent (Fig. 6) [40,72]. A “grafting and coating method” developed in our lab is to covalently graft protein molecules on material surface first, then a protein layer was further physically coated (Fig. 7). This method was used to create a stable collagen layer on PLLA surface to improve its compatibility for chondrocytes [73]. Furthermore, by mixing basic fibroblast growth factor (bFGF) in the collagen solution, proliferation of chondrocytes on the PLLA surface was significantly improved [74]. Layer by layer technique, which has been applied commercially for heparinization of anticoagulant material surface for a few decades, received an extensive renewed research interest in area of biomaterial surface modification in recent years [75]. In our lab, negatively or positively charged biomacromolecules were deposited onto biomaterial surfaces by the LBL self-assembly process (Fig. 5 middle part) [76,77]. To introduce initial charges on the material surfaces aminolysis [76] or poly(ethylene imine) (PEI) treatment [77] were used. By contrast, photo-grafting of PMAA or...
hydrolysis of polyesters can create a negatively charged surface. Hence, the initially assembled substances should be reversed too. A big advantage of the LBL method is its friendly preparation conditions, which are especially suitable for biodegradable polymers and biomolecules. For example, acid fibroblast growth factor (aFGF) was incorporated into the bioactive thin films on a polystyrene surface via the LBL technique. The aFGF built in the multilayers obviously enhances fibroblast proliferation and viability, and regulates the secretion of collagen type I and IL-6. The bioactivity of the multilayers can be largely preserved when stored at −20 °C even for 3 months, while diminished in vitro for few days [78].

2.4. Surface micro- and nano-patterning

In addition to chemical surface modification, a very important approach to manipulate cell behaviors on biomaterial surfaces is surface micro- and nano-patterning. Based on extensive researches in the last decades, it is now well known that micro- or nano-scaled surface patterns, either topographic or chemical, have significant influences on cell behaviors in terms of cell shapes and migration, and protein synthesis and gene expressions [6, 56, 79–81]. Surface patterning of biomaterials are mainly based on lithography which is routinely employed in microelectronics industry to fabricate micro- or nano-patterns on silicon wafer. The topographic surface pattern produced on a silicon wafer can be easily replicated onto a polymeric material surface to form regular physical topographies like ridges, grooves, pillars and dots, etc. Grooves and ridges are among the most studied topographical patterns related to cell morphology control, of which excellent reviews have been written [82, 83]. Microgroove/ridge surfaces have shown significant control over cellular behaviors. The most important phenomenon is that the cell spreading, alignment, and migration can be oriented along the grooves/ridges. One theory that accounts for this is called “contact guide effect”, in which cell integrin receptors in focal contact transfer the variable degrees of tension or compression into the cytoskeleton, and cell stretch receptors subject to these stresses will be activated and reorganize the cytoskeleton according to the surface topography [82]. Another explanation [84] is that the changes of surface free energy due to the edges and disruptions may be the reason for the cell orientation. The cellular response to the surface topographies is also influenced by the dimensions of the surface features, ranging from nano, submicron to micro-scales, depending on the cell types, cell–cell interaction, as well as substrate composition and topography type. In addition to cell morphology, other cell behaviors such as cell adhesion and differentiation are also affected by the material surface patterns. It was found that rectangular ridges with 5 μm width (1–5 μm high) enhanced the adhesion of a kind of marine spore, while decreased the adhesion of endothelial cells [85]. Micro grooves with depths of 0.5, 1.0, and 1.5 μm and widths of 1, 2, 5, and 10 μm on PLA or PS surfaces were found to enhance mineralized ECM production and cell alkaline phosphatase activity of rat bone marrow cells than on smooth surface [86]. In addition to grooves and ridges, micro-pillars with 6 μm in height and 5, 10, 20, and 40 μm in diameter on PDMS surface were found to promote human bone marrow-derived connective tissue progenitor cells’ spreading and adhesion [87]. Polyimide (a kind of photoresist) micro-pillars on glass and micro holes in polyimide film prepared by UV photolithography also affected cell spreading and migration [88].

Besides physical topography patterns, chemical patterns were also fabricated to affect cell behaviors. Microcontact printing (μCP) developed by Whitesides’s group enables translation of topographic surface patterns to chemical surface patterns on biomaterial surfaces [80]. Typically, a PDMS stamp with desired physical surface patterns is coated with a solution containing the substance to be printed, such as adhesion proteins. By contacting the stamp and the substrate, the substance can be printed on the substrate with the same patterns of the PDMS template. Physical interactions such as the strong adsorption between proteins and hydrophobic surfaces or chemical reactions such as –SH and gold surface can be used in μCP to maintain the stability of the micropatterned surfaces. The patterning of proteins using μCP on biomaterial surfaces is an effective approach to control cell adhesion, spreading, orientation, and to obtain cell micropatterns. Singhvi et al. [80] imprinted the hydrophobic compound HS(CH2)13CH3 on a gold surface with a micropatterned PDMS stamp, then hydrophilic thiol compound HS(CH2)11(OCH2)6OH was reacted with the remaining gold surface. The selective adsorption of laminin on the hydrophobic domain can be used to obtain rectangular islands of laminin with different sizes to control the cell extension degree. Fibronectin islands were also imprinted on the gold surface using the same method, and similar results on endothelial cells were found [79]. Kam et al. [89] prepared a honeycomb-like hexagonal micropattern of polylysine-conjugated laminin on a borosilicate glass surface using μCP technology. The hippocampal neurons selectively adhered to the polylysine-conjugated laminin area, and could be guided with high precision. Self-assembled monolayer (SAM) is an important technology to be combined with μCP to prepare surfaces with precise chemical or physical patterns. SAM of amphiphilic molecules formed by hydrophobic interactions as the phospholipid in cell membranes has been used to form surface patterns. Zhang et al. [90] prepared patterns of adhesion peptide (RADS) on the gold surface using a combination of a self-assembly oligopeptide monolayer and microcontact printing. The Microscale RADS tracks were able to guide cell adhesions and orientations precisely.

3. Characterizations of biomaterial surfaces

3.1. Spectroscopic techniques for identification of surface morphology and chemistry

Since the significant influence of the topographic micro- or nano-scaled surface features on cell behaviors, characterization of biomaterial surface morphology is of critical important. The most universal equipment to observe biomaterial’s surface morphology is SEM. The resolution of SEM, however, is usually in micro-scale in the direction vertical to the material surface. In contrast, AFM has much higher resolution in this direction and can detect easily surface ridges or pits of several nanometers in
height or depth. Thus, AFM is becoming a broadly used state-of-art facility to study the biomaterials topography in nano-scaled level and its affect on cell behaviors [91,92]. Not only the biomaterial surface, but also the cell morphology has been observed by AFM to obtain high resolution cell membrane shapes which have been frequently obtained by SEM [93,94]. In addition to the high resolution, another unique advantage of AFM over SEM is its ability to be used in aqueous condition [95], which is exactly the real environment when the biomaterials are used.

Attenuated total reflectance Fourier transform spectroscopy (ATR-FTIR), X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (TOF-SIMS, or simply, SIMS) are among the most widely employed spectroscopic techniques to reveal the surface chemical structure of polymeric biomaterials. Detailed discussion of the mechanisms of these spectroscopic techniques can be found in many text books or monographs so will not be repeated here, but a discussion will be given on their effective sampling depth, i.e. surface specificity. Surface specificity of a spectroscopic technique is a very important parameter which should considered for interpreting the results correctly.

Both single reflection ATR and multi-reflection ATR are widely applied in biomaterial surface analysis. In both models, total reflection occurs on the interface between the sample and the internal reflection element (IRE) made from inorganic crystals like germanium, ZnSe and diamond. Instead of reflecting exactly at the very surface of the IRE, the incident IR will enter into the sample in a form of electromagnetic wave with an exponentially decreasing magnitude and with a penetration depth from several hundred nanometers to more than 1 μm. This kind of electromagnetic wave is called evanescent wave, which has a sampling depth (d) depending on the wavelength and refraction index of both the IRE (n₁) and the samples (n₂), as described in the following equation [96]:

\[ d = \frac{\lambda}{2\pi(n_1^2 \sin^2 \theta - n_2^2)}^{1/2} \]

where λ and θ represent the wavelength of the incident light and the incidence angle, respectively. From this equation, it can be found that the penetration depth of ATR-FTIR has the same magnitude order as the wavelength of infrared light, ranging from several hundred nanometers to more than 1 μm. Therefore, ATR-FTIR is not a very surface specific analysis technique because the ATR-FTIR signal is actually a combination of both the surface and the underneath substrate. Although ATR-FTIR has been used for monomolecular layer analysis, this is only practical when the molecular monolayer is formed on substrates like inorganic crystals [97], silicate [98], silicon oxide and silicon [99], etc., of which the IR absorptions do not overlap with that of the molecular monolayer. In the case of biodegradable polymers like PLA or PCL, the bulk phase’s IR adsorption (for example, 1750 cm\(^{-1}\) for –CO–O–) is so strong that characteristic peaks (1650 and 1550 cm\(^{-1}\) for –CO–NH–) of the surface immobilized polyacrylamide or proteins often cannot be observed by ATR-FTIR. Those grafted layers usually have a thickness from only several to tens of nanometers, comparing to several hundreds of nanometers’ sampling depth of the ATR-FTIR. In these cases, XPS, with much smaller sampling depth, can detect the existence of the grafted layers by observing the N1s peaks.

Compared with ATR, XPS has a much smaller sampling depth (<10 nm) and is a more surface specific analysis technique because the inelastic mean free path (λ) of the X-ray excited photoelectron in polymer matrices is typically 2–4 nm. Although the incident X-ray can go deep into the sample surface (~1 μm for polymeric materials), only the photoelectrons within 3λ (<10 nm) of the surface can escape from the sample. The sampling depth of XPS can be further controlled by changing the take off angle of the photoelectrons, or to say, the orientation angle of the detector. The lower the take off angle, the more surface sensitivity of the measurement can achieve.

One technique with even higher surface specificity (sampling depth 1–2 nm) than XPS is TOF-SIMS or simply SIMS. SIMS is a mass spectroscopy-based technique for analyzing elemental and chemical compositions of the outermost molecular or atomic layer of a solid surface. Only several atomic layers on the sample surface can be analyzed, leading to a high surface specificity. In the last 20 years SIMS has progressed from a novel research methodology to an analytical tool for determination of the polymer surface chemistry in terms of biomedical importance [100–103]. The material surface is bombarded with a beam of energetic ions, usually argon or gallium. When the ions hit the surface, atoms, clusters and molecules will be removed from the material surface in a process called sputtering. A fraction of the sputtered particles will be ionized to produce secondary ions, which can be accelerated to a constant kinetic energy and then allowed to pass a certain distance in a field free environment before they are collected on an intensity sensitive detector. The time of flight of the ions is correlated with individual masses. The positively charged secondary ion fragments are often characteristic of chemical structures on the material surface and can provide significant information for the surface. There are two kinds of TOF-SIMS that have been developed, static and dynamic. Static TOF-SIMS uses a low energy primary ion beam (10⁻⁹ A cm⁻²) to scan the sample surface to obtain a “static” surface analysis. In a dynamic SIMS a high-energy primary ion beam (1 A cm⁻²) and corresponding short time is applied on the sample to erode away the material surface continuously and to record the real time signal simultaneously. The signal is plotted against the depth of the sample to obtain a high resolution depth (on the level of several nanometers), profiling the chemical compositions/structures from surface into the bulk of the sample.

Finally, it should be noted that some techniques like SEM, XPS and TOF-SIMS and SEM require high vacuum to work so that the information obtained might not be truly representative of the actual liquid–solid biomaterial surface. In contrast, those techniques which do not need high vacuum conditions like ATR-FTIR and AFM can be used in aqueous environment to obtain valuable information.

### 3.2. Colorimetric method to determine surface density of functional groups

ATR-FTIR, XPS and SIMS are powerful techniques to understand surface chemistry quantitatively or qualitatively. A complement approach to measure the amount of surface func-
tional groups on a biomaterial surface is colorimetric method, particularly when a large amount of functional groups exist on the surface. Several colorimetric methods have been developed to effectively measure the surface density of carboxyl groups and amino groups on polymer surfaces, based on either ion exchange mechanism or particular chemical reactions, as will be discussed later. The main disadvantage of the colorimetric methods, however, is that they are far less sensitive than XPS and SIMS, and thus is only useful when a large amount of surface functional groups are present. Further, when the colorimetric method is based on ion exchange mechanism, functional groups have to be subjectively assumed to be able to bind equal molar amount of dye molecules.

Rhodamine 6G (Rd6G) can be used to determine trace amount of carboxyl groups [104]. The Rd6G is first extracted with toluene from alkaline solution to form an orange-colored R6G/toluene solution. Surface carboxyl groups at very low concentration can change the color of the R6G/toluene solution from orange to pink, producing new absorbance at 513 nm. Another method to determine surface carboxyl density is toludine blue (TBO) colorimetry [63,105,106]. TBO is a positively charged molecule that can combine with a carboxyl group in alkaline solution to form a stable electrostatic complex through ion exchange mechanism, as shown in Fig. 8. The TBO molecules immobilized on surface can be detached by dissolving in acetic acid or other organic solvents. The amount of the –COOH groups can be calculated by the assumption that they combine with TBO stoichiometrically [106].

Similarly, methyl orange (MO) is a negatively charged dye and can combine with positively charged amino groups on material surface under acidic condition by ion exchange mechanism, as shown in Fig. 9 [107]. The combined MO molecules can be dissolved in basic solution or organic solvents, thus the MO amount can be determined by optical absorption. By assuming a combination ratio of 1:1 between MO and –NH₂, Hartwig et al. measured the amino group density on ammonium plasma treated polymer surface, which was as low as several nano-mole per square centimeters [107]. We used ninhydrin method to determine the surface density of amino groups on aminolyzed PCL surface [66]. Two ninhydrin molecules can react with one primary amino group to be reduced to a purple-blue colored product, of which the absorbance at 570 nm was measured.

![Fig. 8. Quantitative analysis of –COOH group on material surface: (a) in basic solution (pH ∼ 10), positively charged TBO form complex with –COO⁻; (b) in acidic solution (pH < 2) or organic solvents, the TBO molecules are released from the material surface; (c) molecular structure of TBO.](image)

![Fig. 9. Quantitative analysis of amino group on material surface. (a) In acidic solution (pH ∼ 5), negatively charged MO form complex with –NH₃⁺; (b) in basic solution (pH > 10) or organic solvents, the MO molecules are released from the material surface; (c) molecular structure of MO.](image)
3.3. Thickness and molecular weight of the grafted polymer layer

Although techniques such as X-ray and neutron scattering [108,109], ellipsometry [20,110,111] and ATR [112] have been developed to detect thickness of ultra thin solid films, very few work has been reported on measuring layer thickness on a real polymeric biomaterial surface due to the rough nature of both the substrata and the grafted layers, nor has the average molecular weight of the grafted polymer been characterized. Conventional radical grafting copolymerization on material surface produces grafted chains with high polydispersity. Average molecular weight of the grafted polymer chains can be calculated by dividing the amount of grafted polymers by the grafting point density. However, the grafting point density is difficult to measure. Steffens et al. assumed that the PMAA chains grafted on PLA surface had a grafting point density of one PMMA chain per nm$^2$ [29].

Ejaz et al. and Yamamoto et al. grafted poly(methyl methacrylate) (PMMA) onto a silicon wafer surface using living radical polymerization [113–117]. The living radical polymerization can produce grafting polymer chains with relatively monodisperse ($\text{Mw}/\text{Mn} < 1.3$) molecular weight, so that the relationship between the grafted layer thickness and its molecular weight can be established. The layer thickness and the molecular weight were characterized by ellipsometry and AFM, and gel permeation chromatography (GPC). The correlation between the thickness of the grafted PMMA ($T$, nm) and the molecular weight is $T \approx 7.6 \times 10^{-4} \times M$. A grafting point density of 0.45 chain/nm$^2$ was finally calculated. Theoretical calculation of a fully extended PMMA chain gives a chain length ($L$, nm) of $L \approx 25 \times 10^{-4} \times M$. Hence, it can be concluded that the PMMA chains are far from being fully extended.

3.4. Protein densities on biomaterial surfaces

Earlier studies showed that XPS can provide information about the coverage and thickness of proteins adsorbed on a surface. For example, XPS can distinguish whether the adsorbed protein layer exists in a continuous manner or as a patchy film [118,119].

The protein amount immobilized on biomaterial surface can be effectively measured by radio labeling of the protein. Radio labeling of protein by $^{125}$I is a straightforward and sensitive method for protein amount analysis. The radioactivity measured by a scintillation counter is proportional to the total mount of the protein. Ninhydrin [120] and bicinchoninic acid (BCA) [121] methods can be easily performed in an ordinary lab. In ninhydrin method, the protein-immobilized material was first immersed in strong acidic solution to completely hydrolyze the protein into amino acids. After neutralization with NaOH, the amino acids reacted with ninhydrin to form a colored product with a maximum absorbance at 570 nm. BCA method (Fig. 10) is an effective approach to determine the amount of the immobilized protein on biomaterial surface, with available commercial kit such as BCA Protein Assay Kit by Pierce Company. In this method, Cu$^{2+}$ ions are reduced to Cu$^{+}$ ions by protein, and one Cu$^{+}$ ion combines with 2 BCA molecules to give a purple colored complex which exhibits a strong absorbance at 562 nm (Fig. 10). The reaction can proceed regardless of the state of proteins, i.e. both free state and immobilized proteins can reduce the Cu$^{2+}$ to Cu$^{+}$ ions. Therefore, unlike the ninhydrin method, there is no need to remove the immobilized protein molecules from the material surface.

A quartz crystal microbalance (QCM) (or piezoelectric microbalance) measures mass by measuring the change in frequency of a piezoelectric quartz crystal when it is disturbed by the addition of a tiny mass such as proteins or any other tiny objects intended to be measured. QCM with dissipation (QCM-D) technique can estimate changes in both the mass and the viscoelastic constant for the adsorbed layer through measurements of frequency and dissipation [122]. QCM and QCM-D can work under both vacuum and liquid environment thus making it useful to determine adhesion of proteins on polymers. Lord et al. [123] used QCM-D to study adsorption of biomolecules on the PHHEMA based hydrogels and found most biomolecules can

![Fig. 10. (a) The reaction mechanism of BCA Protein Assay Kit; (b) the structure of the bicinchoninic acid, or BCA; (c) chemical structure of the Cu$^+$-BCA complex.](image-url)
be adsorbed on the hydrogel surfaces causing increases in mass and dissipation upon adsorption. With use of QCM-D technique, Welle [124] found that the quantity and viscosity of surface bound albumin on polystyrene can be lowered after the substrate is UV (185 nm) treated.

Surface protein thickness can be characterized by ellipsometry [20,110,111]. The technique is based upon measurement of changes in polarization state of a reflected light from its incident light. When a monochromatic linear polarized light is reflected by a smooth surface, the polarization state will be changed, and will be further changed if a protein layer exists on the surface. The polarization state of the reflected light is related to many parameters including the protein layer thickness, which can be calculated through fitting the changes of polarization with a mathematical model using a computer. However, a great limitation of ellipsometry is that only proteins on very smooth surface with strong reflection ability and different refractive index from that of proteins (∼1.45) such as silicon or silicon dioxide can be quantified. Thus, the technique is not practical for polymeric biomaterial surface analysis. This limitation also exists for another important surface protein analysis technique, surface plasmon resonance (SPR) [112,125]. In the case of total internal reflection, the incident electromagnetic field penetrates a short (tens of nanometers) distance into the medium of the lower refractive index creating an exponentially detenuating evanescent wave. If the interface between the two media is coated with a thin layer of metal (gold), and incident light is controlled as monochromatic and p-polarized, at a particular incident angle the intensity of the reflected light is reduced due to the energy transfer between the evanescent wave and surface plasmons, called surface plasmon resonance. The resonance will be influenced by biomolecules adsorbed onto the thin metal film. Linear relationship is found between resonance energy and mass concentration of the biomolecules such as proteins or DNA. Therefore, SPR is an optical technique to investigate biological interactions like protein adsorption process and is capable of real time in situ analysis to define protein adsorption and desorption rate [125].

3.5. Bioactivity of the proteins immobilized on biomaterial surfaces

The bioactivity of the immobilized proteins on biomaterial surfaces is another importance issue. Bioactivity of proteins can be characterized using the specific bio-recognition process between antibodies and antigens. If the natural conformation (bioactivity) of the proteins on the biomaterial surface is preserved, its specific bio-recognition binding with its antibody will be available. Thus, the bioactivity of the proteins immobilized or adsorbed on biomaterial surface will be expected to correlate with the total amount of the bonded corresponding antibodies, which can be measured quantitatively by enzyme-linked immuno sorbent assay (ELISA). Tziampazis et al. measured the bioactivity of fibronectin adsorbed on surfaces with different PEG concentrations by ELISA assay using anti-fibronectin antibody. It was found that the degree of cell attachment varied with the PEG concentrations in a manner similar to the dependence of fibronectin bioactivity on the PEG concentrations [28].

Circular dichroism spectroscopy (CD) is a routine technique to study protein conformation (secondary structure) [126,127]. Alpha-helix, beta-sheet, and random coil protein structures generate different absorbance and refractive indices to both right circular and left circular polarized lights, resulting in different CD spectra. The approximate fraction of each secondary structure type can thus be determined by analyzing the total CD spectrum by referencing spectra corresponding to each structural type. Although from a CD spectrum it is not able to determine exactly whether a protein remains its bioactivity, the denaturing degree of the surface immobilized protein can be estimated by comparing the CD spectra of the native and the surface immobilized proteins. Using CD method, Tanaka et al. [127] found that plasma proteins adsorbed on poly(ethylene glycol) (PEG)-containing polymer surface had less change of the CD spectroscopy as on other materials.

One important application of SIMS technology introduced in Section 3.1 is to study the composition, conformation, orientation and denaturation of biomacromolecules on biomaterial surfaces [128,129]. Compared with XPS, SIMS is more chemical selective due to the basis of the mass spectroscopy. For protein-immobilized surface, the ion fragments (such as amionium ions) produced by the bombardment of the surface are characteristic of different specific amino acids. Therefore, the intensity ratio of different amino acid mass fragments provides useful information for the identification of the immobilized proteins. Moreover, the small sampling depth of 10–15 Å offers the potential to gain a detailed understanding of the conformation and orientation of the protein molecules. Most proteins of interest have dimensions of 1–10 nm. The static SIMS spectrum of an adsorbed protein represents an amino acid assay of the outer 10–15 Å of the protein. So the relative intensities of the amino acid fragments detected in the static SIMS spectrum are sensitive to the orientation of the protein on the surface and its degree of conformational alteration. As a protein adjusts to the surface and changes its conformation or orientation, new regions of the protein with different amino acid compositions will be exposed to the static SIMS sampling depth and be detected. Using the static SIMS technique, Tidwell et al. studied the conformation change of albumin and fibronectin molecules adsorbed on different substrates [129].

A technique that can provide also significant insights into the behavior of the immobilized protein molecules is electron paramagnetic resonance (EPR, also known as ESR, electron spin resonance) spectroscopy. The immobilized protein molecules are firstly labeled with spin labels (radical labels) which generally belong to a nitroxide type. The EPR spectra of the nitroxides are especially sensitive to molecular mobility. Under fast, isotropic motion, as normally occurs with nitroxide molecules in solution, a sharp three-line EPR spectrum is recorded. However, if the motion becomes hindered, as for example occurs if the spin labels are bound to the immobilized protein molecules, asymmetric line broadening occurs. Therefore, the shape of the EPR spectrum of the spin labeled protein molecules indicates the mobility and bioactivity of the labeled protein domains. Spe-
cific sites of proteins can be selectively bound with the spin labels. A variety of spin labels for specifically labeling protein sites is either commercially available or can be often readily synthesized. Therefore, the EPR spin-labeling technique has considerable potential for clarifying the behavior of specific sites of proteins immobilized on biomaterial surfaces [130,131]. Butterfield et al. studied the bioactivity of the immobilized enzyme on polysulphone surface using EPR technique. The active site of papain was labeled with 1-oxyl-2,5,5-tetramethyl-2-pyrrolidone-3-methyl (MTS). It was found that the enzyme’s catalytic activity is proportional with the mobility of the active sites attained by EPR spectrum analysis. Furthermore, the accessibility of small molecules to the active sites of the enzyme was also studied using K$_3$Fe(CN)$_6$ by broadening the EPR signals [132].

4. Cytocompatibility evaluation

4.1. Cell proliferation (number) measurement

The most straightforward way for cell number measurement such as cell adhesion and proliferation is to count the cell number after the cells are detached from the material via trypsinization. One drawback is its large system error and standard deviation, because sometimes the cells are hard to be detached completely from the rough biomaterial surfaces, especially from 3-D scaffolds. Many colorimetric methods have been developed for cell number determination, among which the methods based on cell’s metabolic viability test are widely employed. Methylthiazoletetrazolium (MTT) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) are reagents that can be reduced by the dehydrogenase enzymes in cell plasma to form a purple colored formazan product. The total amount of the purple colored product can be quantified by optical absorption, giving the total reduction ability of the dehydrogenase enzymes in the cell population. Commercialized MTT and MTS kits are available, such as CellTiter 96 AQueous One Solution provided by Promega. The advantage of the MTT and MTS method is that the cells need not to be detached from the material surface prior to the measurement. The drawback is that the cell number is not necessarily proportional to the total metabolic viability because the amount of the dehydrogenase enzymes per cell, i.e. the metabolic viability of every cell, may not be constant.

Another type of colorimetric methods to determine the cell number is based on total DNA amount measurement. PicoGreen (excitation at 470 nm and emission at 525 nm) and Hoechst 33258 (excitation at 470 nm) are two commonly used fluorescence dye for double strand DNA, which become intensely fluorescent upon binding with the nucleic acids. The fluorescence enhancement of PicoGreen upon binding double strand DNA is more than 1000-folds. One commercial available product for PicoGreen staining is Quanti-It™ PicoGreen® dsDNA Reagent and Kits provided by Molecular Probe. The drawback of the DNA amount measurement method is that the cells must be detached and lysed through a laborious and intensive process. Radio labeling by $^{3}$Hthymidine can measure increased cell numbers precisely. When a new cell is produced by the cell proliferation, new DNA molecules must be synthesized. Upon adding the radioactive $^{3}$Hthymidine, new DNA molecules containing the radio label will exist in the nucleus. After removing the free $^{3}$Hthymidine molecules, the radioactivity of the sample will show a strong linear relationship with the increased cell numbers. The method cannot measure the total cell number because the original cells do not have $^{3}$Hthymidine. Other drawbacks include healthy risk associated with the radioactivity and the need of a scintillation counter instrument. To overcome the problem of radioactivity, Roche Company developed a cell proliferation ELISA kit which uses 5-bromo-2’-deoxyuridine (BrdU) as an analogue of thymidine. By supplemented into the cell culture system, the BrdU is incorporated into the newly synthesized DNA molecules, whose amount can be analyzed by ELISA using anti-BrdU antibody.

4.2. Cell imaging

Imagining of cell morphology and distribution on/in biomaterials is an important issue in tissue engineering because it is often closely related to cell phenotype and function. For example, spreading endothelial cells (ECs) may cover the foreign material surfaces to isolate the blood and the materials, leading to the reduced thrombosis and immunoreactions. In addition to the conventional light microscopy and SEM, fluorescein diacetate (FDA) [133] or chloromethylfluorescein diacetate (CMFDA) [134] staining provides a convenient method to observe viable cell morphology without cell fixing. The non-fluorescent lipophilic dyes like FDA and CMFDA can penetrate through cell membrane and be hydrolyzed by the cell’s hydrolyase enzyme into hydrophilic fluorescent products that cannot easily diffuse out of the cells. Hence, cell morphology can be easily observed under fluorescent microscopy or confocal laser scanning microscopy. CMFDA has no negative effect on cell growth, thus can be used to monitor the long-term cell culture. Recent studies have demonstrated that atomic force microscopy (AFM) is a potential tool for studying important dynamic cellular processes in real time [93,135–137]. Continuous imaging of individual cell in physiological buffer was carried out for several hours without damage of the cell as judged by its persistent undisturbed morphology. Dynamic events such as protrusive activity, filopodia/lamellipodia spreading and retracting and mitotic cycle, were observed in time course. Fig. 11 shows a time series of AFM deflection images of a single cell [93]. In Fig. 11(a), which was recorded 50 min after seeding, the cell appeared not yet to be fully spread. Numerous filopodia (arrow indicated) were stretched out from the lower edge of the cell to form flat lamellipodia during the next 10 min. The lamellipodia were fully developed after 15 min (Fig. 11b). Simultaneously the upper part (arrowhead indicated) was slightly retracted and increased in height. After 35 min the lamellipodia were retracted and the cell appeared more rounded (Fig. 11c). 20 min later the cell was found to be spread even wider [Fig. 11(d)]. AFM can also investigate the mechanical properties of the cytoskeleton by measuring the cell’s elasticity.
Immunofluorescence is another most important methodology for characterizing cellular structures (e.g. nucleus, lysosomes, cytoskeletons), which can also visualize the dynamic processes in the cell. The principle underlying this technique is the ultra high affinity between two molecules, e.g. antigen/antibody. One makes a distinction between direct immunofluorescence and indirect immunofluorescence. With direct immunofluorescence, the component, which specifically recognizes a cellular target molecule, is directly coupled with a fluorescent dye. Indirect immunofluorescence works according to the sandwich method. An antibody first binds to the cellular target molecule (antigen) and, in a second step the antigen–antibody complex is detected with a second, dye-coupled antibody. Hoechst 33342 (excitation by UV and emission at 470 nm) was used to stain the nuclei of living cells. 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, excitation by UV and emission at 470 nm), Hoechst 33258 and ethidium bromide (excitation at 545 nm and emission at 610 nm) were routinely used to stain the nuclei of living cells. Immunofluorescence stain of F-actin, β-tubulin, vinculin was carried out to study the cell morphology too. [138] In Fig. 12 (A, A′), mouse B16F1 melanoma cells (B16 cells) were cultured on a homogenous fibronectin substratum prepared with microcontact-printing technique. Fluorescence staining for vinculin (Vin, green) and actin (red) on a patterned substratum of 0.6 μm² fibronectin dots (FN, blue). Vinculin has accumulated in areas of the cell overlying ECM dots. Actin fibers terminate in most of these adhesion sites, indicating functional contact sites. In Fig. 12 (C, C′) cells were stained for focal adhesion kinase (FAK, green) on a patterned substratum of 1 μm² FN dots (red).

At the end of last decade, quantum dots, a kind of semiconductor nanocrystals were used to replace the conventional dye molecules because they have a narrow, tunable, symmetric emission spectrum and are photochemically stable [139–143]. Green fluorescence protein (GFP) gene fusion can be utilized to visualize living cells. In fact, the GFP gene is an excellent reporter gene in gene transfection and now is widely used in study of gene delivery. The GFP gene can be transected to cells and expressed by the cells to produce GFP in their cytoplasm. The fluorescent GFP can be detected in living cells without selection or staining and it can be fused to other proteins to yield fluorescent chimeras. Various GFP-tagged genes have been constructed to express the GFP associated functional proteins when transferred into living cells [139,144]. Recently, with the advent of GFP and its derivatives in cell biology, molecular dynamics imaging was developed to analyze dynamic processes in living cells. This method has three major advantages: high spatial resolution (about 250 nm), high temporal resolution (few seconds) and extended image sequences (more than 100 frames).
Fig. 12. B16 cells were cultured for 1 h on patterned ECM substrata and labeled for focal adhesion molecules and ECM proteins. (A, A’). Fluorescence staining for vinculin (Vin, green) and actin (Act, red). (B, B’). Cell stained for vinculin (Vin, green) and actin (red) on a patterned substratum of 0.6 μm² fibronectin dots (FN, blue). (C, C’). Cell stained for focal adhesion kinase (FAK, green) on a patterned substratum of 1 μm² FN dots (red). Scale bars: 10 μm.

The method opens new vistas for studying cytoskeletal dynamics and has demonstrated the potential to reveal new cellular behaviors [138, 145]. Fig. 13 presents the cell spreading dynamics on fibronectin dots of 0.1 μm² [138]. The images of a time-lapse sequence were recorded for a B16 cell expressing β3-integrin-GFP (β3-GFP) and growing on a patterned substratum of 0.1 μm² fibronectin dots. Dots were visualized by mixing the fibronectin with fluorescently labeled BSA. Since β3-GFP cells display homogeneous background fluorescence, they were used in these assays to reveal cell morphology. The cell was highly motile but did not spread. A few minutes after initial contact, a dot (arrows in A–F) was first stretched (arrow in C), removed from the substratum (arrowhead in D) and then internalized into the cell (arrowheads in E and F). This behavior results in a rearranged dot pattern after a cell has migrated over that area.

4.3. Cell migration

Cell migration depends on the dynamic interplay between the cells substrate and cytoskeleton. Firstly, the cell develops a protrusion of its leading edge to form a lamellipodium. Secondly, after formation and fixation of the lamellipodium, the cell uses adhesive interactions to generate the traction and energy required for cell movement. The last step of the migratory cycle is the release of adhesions at the rear of the cell followed by its detachment and retraction [146, 147]. It remains as yet largely unexplored as to how these processes are coordinated and regulated as an integrated system. As far as we known, cell motility across a substratum strongly depends on the biophysical nature of adhesive interactions. The integrins, which are heterodimeric transmembrane proteins, bind to the ECM molecules as well as to cytoskeletal components and certain intracellular
signaling molecules within the cell [148,149], providing signals from the ECM in addition to structural linkages [150]. Integrin-mediated signals regulate a variety of cellular functions such as proliferation, differentiation and migration [151,152]. ECM binding leads to the activation of second messengers that are also activated via the binding of growth factors to tyrosine kinase receptors [153,154]. Recent work has shown that synergy effect occurs between growth factors- and ECM-mediated events in regulating some of these signaling pathways and cell functions [155,156].

It has been shown both theoretically and experimentally that the average speed of cell locomotion typically exhibits biphasic dependence on the strength of cell substratum adhesion. For a number of cell types, the maximal cell migration speed occurs at an intermediate value of cell–substrate adhesive strength [55,157–160]. Systematic variation in cell–substratum adhesion strength has most commonly been achieved by variation of the surface density of ECM proteins [161], thus changing the number or strength of bonds between the adhesion receptors and the extracellular components. Comparable effects have been achieved in a three-dimensional system by adding systematically varied amounts of fibronectin to collagen gels [162]. Cell–substrate adhesion strength can also be altered by addition of soluble growth factors [161]. Some antiadhesive extracellular matrix proteins such as tenascin, thrombospondin, muscin and proteoglycans also play roles in cell migration [163].

In the absence of other stimuli such as chemical, electrical or other oriented signal gradients, cells moving on a flat surface show random walk [164]. However, cell migration can be oriented by chemical, physical and other stimulus. Zhao et al. [165] observed that cells reacted to chemotaxis including peptide, cytokine and growth factors in a collagen gel model. Cells movement followed the chemotactic gradient to the source over a distance up to 500 μm and time up to 8 h. Cao et al. [166] determined the minimum nerve growth factor concentration gradient for effective guidance of PC12 cell neurite outgrowth. Higher gradients were effective for guidance. At a nerve growth factor concentration of 995 ng/ml, the PC12 cells’ receptors were saturated, thereby limiting the maximum effective distance for guidance to less than 7.5 mm in response to a diffusible nerve

![Fig. 13. Dynamics of cell spreading on fibronectin dots of 0.1 μm². For detail see the text. Time is given in minutes and seconds. Scale bars: 10 μm.](image-url)
growth factor cue. DeLong et al. [167] built a covalently immo-
obilized bFGF gradient hydrogel scaffold. They demonstrated
that smooth muscle cells migrated differently, up the concen-
tration gradient, on bFGF-gradient hydrogels compared to control
hydrogels. Smith et al. [168] claimed also that cells showed
an accelerated migration on linear fibronectin gradients on a
polymer surface.

Cell movement is also regulated by micro- and nano-scale
topographies on substrate surface such as grooves and fibers
[82]. Cells on topographies such as grooves often show highly
oriented movement, which in many cases is one-dimensional.
On the opening logarithmic spiral grooves, cells migrated cen-
trifugally, not along the groove, but by forming chords from one
part of the groove edge to another, usually crossing from the
inner side to the outer side of the groove further out on the spiral
[169]. The cells on grooves or ridges often show very narrow
lamellipodia. Thus, those in grooves or on ridges must exert rel-
atively greater tractive effort per unit area than those on a planar
surface. Focal contact and vinculin are aligned over or close to
the groove/ridge contact [170,171], while the actin cytoskeleton
in cells reacting to topography is organized in a way which is
believed to be appropriate for movement [172].

Several techniques have been developed to characterize the
movement of both individual cells and whole populations. The
wound assay, the Teflon fence assay, and the phagokinetic track
assay all measure cell movement on uniform substrates using
information from only the beginning and end of the experiment
[168]. In these techniques, cells migrate from known positions
defined by the system geometry and are fixed for viewing at
the end of the experiment to determine the net distance trav-
elled during the experiment. Video microscopy techniques have
the ability to continuously monitor the cell behaviors during
migration and provide the possibility of tracking discrete cell
motion.

4.4. Cell function analysis

The most important cell function is to secret characteristic
biomolecules that are closely related with corresponding tissue
functions. These biomolecules include ECM structural proteins,
plasma proteins, growth factors, enzymes and glycosaminogly-
cans (GAGs). In native cartilage tissues collagen type II and
GAGs are the important structural substances. Hence, in carti-
lage tissue engineering chondrocytes are frequently checked on
their ability to secret these molecules, using immunostaining,
Western blot or colorimetric methods. For blood vessel tissue
engineering, von Willebrand factor (vWF) is closely related
with blood clotting process and is one of the most important
characteristic proteins expressed by endothelial cells, thus is
often determined by ELISA as a criteria to evaluate endothelial
cell’s phenotype and functions [66,69]. Other marker proteins
expressed by endothelial cells include ICAM-1, VCAM-1,
PECAM-1, IL-1, TNF-α and E-seleltcin, etc. [173–177]. In
bone tissue engineering, alkaline phosphatase, a characteristic
enzyme secreted by the osteoblasts, represents the bone regen-
eration ability of osteoblasts. The alkaline phosphatase activity
can be measured as a function of the ability to hydrolyze p-
nitrophenyl phosphate into p-nitrophenol.

Fig. 14. Schematic diagram of DNA microarray experimental process. cDNAs of both the tested cells (A) and the reference cells (B) are differently tagged and
hybridized simultaneously on one microarray chip, which allows quantitative analysis of expression of thousands of genes in the tested cells at the same time.
In addition to protein expression, cell function should be studied in terms of gene expression too. Techniques such as transcriptase–polymerase chain reaction (RT-PCR) and in situ hybridization are routinely used to analyze whether genes encoding for target makers (characteristic biomolecules) can be found in the mRNA library of the cells. cDNA microarray is a new developed technique to screen expression level of tens of thousands of mRNA messages in a high throughput fashion [178]. In a microarray experiment as shown in Fig. 14, hundreds to thousands gene-specific nucleotides are individually arrayed on a single chip. Total mRNA of the tested cells is extracted, and then translated into cDNA by reverse transcription (RT) process, in which the nucleotides used are fluorescence- or radio-tagged so that the cDNA produced is fluorescence- or radio-tagged. The microarray chip is then hybridized with the fluorescence- or radio-tagged cDNA, allowing the determination of the relative amount of the transcripts in the cells by scanning the chip and analyzing the fluorescence or radio signals. cDNAs of both the tested and reference cells are usually hybridized with one microarray chip simultaneously to compare the gene expression signals between the tested cells and the reference cells. In this case their cDNAs should be differently labeled (Fig. 14).

5. Conclusions

Surface engineering and characterization of biomedical polymers for tissue engineering were reviewed from our particular perspectives. While many methods to increase surface hydrophilicity have been employed to improve material’s cytocompatibility, immobilization of specific protein biomolecules is the most effective way to prepare surfaces with customized cell response. Reactive groups such as –COOH and –NH2 are usually introduced onto material surfaces as coupling sites to covalently attach proteins. Proteins can also be physically immobilized on material surfaces using methods such as “grafting-coating” and “layer-by-layer” self-assembly. Surface chemical composition analysis techniques like ATR-FTIR, XPS and SISM have very different sampling depth, which should be considered in interpreting the results. Colorimetric methods using specific dyes are convenient to quantify the amounts of surface functional groups, surface grafted polymers and immobilized proteins. Surface analysis techniques like QCM, SPR, CD, SIMS and EPR can provide useful information on protein density, conformation and orientation on biomaterial surfaces. Cell behaviors in vitro on biomaterials may be assessed with respect to cell proliferation (number), morphology, cell migration, ECM and specific factor secretion, and gene expression. Cell proliferation can be tested using different methods in terms of cell number, metabolic viability and DNA amount. AFM, immunofluorescence and quantum dot staining are newly developed cell imaging techniques. Cell migration speed is closely related with the interactions between the cell and the substrate, and can be characterized by video microscopy techniques. cDNA microarray is a powerful gene expression analysis technique to screen the expression of many specific genes simultaneously.

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