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Characterization of biomaterials

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7.1 Introduction

A biomaterial is a natural or synthetic material that can be used for different applications such as tissue engineering, bioelectrodes and biosensors, drug delivery, gene therapy, diagnosis of disease, and the improvement of the healthcare. The main characteristic of all biomaterials is their ability to be in the living systems, although the effects of both the biomaterial on the host tissues, and the living system on the biomaterial lead to immunological interactions (local and systemic) and thereby may cause device failure, patient's distress, or even fatality; therefore the biomaterials must be biologically sustainable and biocompatible. Biocompatibility is the behavior of a material in a living tissue that does not cause an adverse effect such as producing a toxic, a physiological reactivity, or an immunological response. Biomaterials can be metals, ceramics, polymers, hydrogels, or composite materials that used vastly in literature for oral and dental tissue engineering or relevant applications (Razavi et al., 2015; Yazdimamaghani et al., 2014; Razavi et al., 2014b; Yazdimamaghani et al., 2013; Razavi et al., 2014a; Heidari et al., 2015). The first challenge in employing the right material for specific application is investigating the chemical, physical, and biological characteristics of it, which is the focus of this chapter.

7.2 Chemical characterization techniques

The determination and analysis of the chemical characteristics and molecular structures of biomaterials often is required for different applications. This section presents an overview of the important chemical characterization strategies and spectroscopic techniques, including infrared (IR), Raman, X-ray photoelectron spectroscopy (XPS), ultraviolet−visible (UV/Vis), nuclear magnetic resonance (NMR), mass spectrometry (MS), and their relative applications.

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7.2.1 Infrared spectroscopy

IR spectroscopy is a commonly used optical technique for identification of compounds and their chemical structure analysis. Diverse functional groups within a molecule absorb IR radiation at various frequencies; therefore IR spectroscopy infers the presence or absence of certain chemical functional groups in a molecule. This technique is nondestructive and can be applied to analyze gaseous, liquid, or solid materials. The energy of the IR photons is not adequate to cause transition of the valence electrons; however, the vibrational and rotational motions are excited in molecules by IR radiation. The spectra are achieved by passing (reflecting) a beam of IR radiation through (from) a material. The resulting spectrum consists of a plot of absorption, transmission, or refection intensity versus wavelength or frequency. The IR can be divided into three spectral subregions; the near extends from 800 to 2500 nm (NIR), the mid from 2500 to 15000 nm (MIR), and the far-IR from approximately 15,000 to 100,000 nm (FIR).

The most common type of IR instrument is the Fourier transform infrared (FTIR) spectrometer owing to its high signal-to-noise ratio, easy to use, and relatively low cost. An FTIR enables measuring all wavelengths at once; therefore the whole spectral information is gathered at the same time (Yu et al., 2009; Kassi et al., 2010). Fig. 7.1 demonstrates the principle operation of an FTIR. As shown, the FTIR is like a Michelson interferometer with the only difference that one of the mirrors is movable and is controlled by a motor. Therefore it is possible to change the time delay between the reflected beams off the two mirrors continuously using

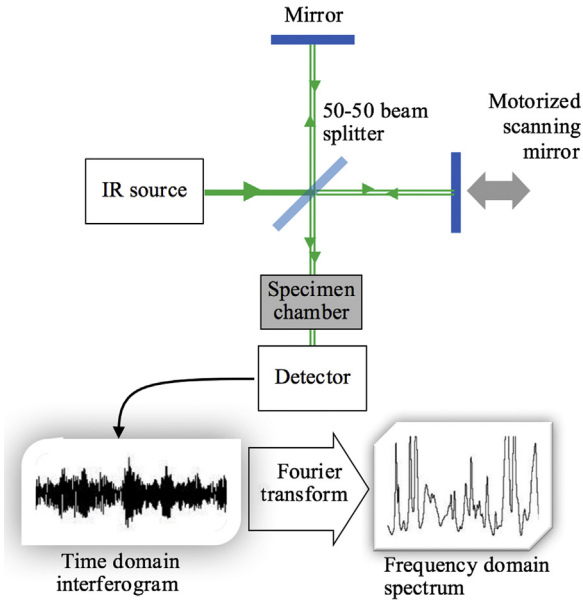

Figure 7.1 The basic configuration of the FTIR spectrometer.

the movable mirror. The specimen is located in front of the detector where the interferogram is recorded. The interferogram data are not useful as it is; however its Fourier transform reveals information about the spectral response of the specimen. The specimen chamber may be in a configuration to send out the transmitted or reflected beam.

FTIR spectroscopy is a valuable tool that is widely used by researchers. As a biomedical example, FTIR has been used for the investigation of the conversion degree in dental composites and the polymerization process (Ahuja and Scypinski, 2010; Araujo et al., 2015; Moraes et al., 2008; Ilie and Durner, 2014). Also, a specific technique named attenuated total reflection FTIR (ATR-FTIR) spectroscopy is frequently employed to characterize biomaterials. The main advantage of the ATR-FTIR is the feasibility of direct assessment without using any pretreatment or destructing the specimen during the preparation procedures (Durner et al., 2012).

7.2.2 Raman spectroscopy

Raman scattering, another nondestructive and noninvasive spectroscopic method and complementary to IR absorption spectroscopy, is employed to assess molecular vibrations and has considerable potential as an analytical technique in the characterization of biomaterials in any state (solid, liquid, or gas). Selection rules that govern the principles of Raman scattering and IR absorption spectroscopy are completely different. Thus there exist certain vibrational mode excitations that are allowed in IR spectroscopy but are forbidden in Raman spectroscopy. The Raman spectra are generated due to the interaction of the photons and the specimen molecules and are collected using optical filters form the scattered light. The Raman spectra contain information about the chemical species, molecular structures, and the conformation of the materials and provide a "fingerprint" for their identification. Generally, in a Raman spectrum, the Raman intensity is plotted versus the Raman shift. The Raman shift is defined as the difference of frequencies between the Raman scattered and the incident light beam. The C−S, S−S, C−C, N═N, and C═C groups are the examples which provide strong Raman bands (Brittain, 2011; Petry et al., 2003; Bazin et al., 2009).

7.2.3 X-ray photoelectron spectroscopy

XPS is a very potent technique, suitable for characterizing the elemental and chemical composition of the very top surface (1−10 nm) of any solid surface. The phenomenon is based on the photoelectric effect that refers to the surface bombardment with X-ray photons. The electron ejection occurs using a monochromatic beam of soft X-rays in an ultrahigh vacuum environment. The electrons are then emitted from the shell of the atoms and their kinetic energy and number are simultaneously measured by detectors. The difference of the photon energy and the sum of the electron kinetic energy and the work function determines the electron binding energy. The work function depends

on both the spectrometer and the material, so it is often treated as an adjustable instrumental correction factor. The resulting XPS spectrum consists of a plot of the detected number of photoelectrons as a function of the binding energy. The binding energy of the electrons is a characteristic of the elements, but it is also affected by the formal oxidation state, the local bonding environment such as the identity of the nearest-neighbor atoms, bonding hybridization to the nearest-neighbor atoms, etc. Therefore XPS is sensitive to the chemical nature of the materials and provides somewhat different results for different chemical binding states.

The use of XPS is limited in the analysis of biological materials as they have relatively short lifetimes in high vacuum and under radiation damage caused by X-ray photons although the ambient-pressure XPS is a current area of development. There are, nevertheless, vast applications of XPS in biomaterials characterizations. Many different studies have underlined potential of XPS to examine and analyze the types of biomolecules, some of which include proteins and peptides, lipids, enzymes, and DNA. XPS enables investigating the extent of the functionality and specific adsorption or binding of these biomolecules onto various surfaces; hence, it helps to develop biosensors, bioarrays, and biofouling controls for medical or dental applications. XPS has been also applied to the analysis of hard tooth tissue in restorative dentistry, including the effects of different treatments and chemical agents for the health of the tooth, and understanding of the mechanisms underpinning the interactions between the biomaterial and the hard tissue (Artyushkova and Atanassov, 2013; McArthur et al., 2014).

7.2.4 Ultraviolet–visible spectroscopy

UV–Vis spectroscopy, one of the earliest characterization techniques, can be utilized to measure the absorbance of UV to visible light by a material as a function of the wavelength. The UV region extends from approximately 190–350 nm and the visible region from 350 to 800 nm. The absorption happens duo to the electronic transitions from the ground state to the excited state and its magnitude depends on the Beer–Lambert law:

$$A = abc$$

In which A is the absorbance, a is the wavelength-dependent absorption coefficient, b is the path length through the solution in an analytical cell, and c is the molar concentration of the absorbing analyte.

This nondestructive spectroscopic method offers both qualitative and quantitative information about a liquid sample. According to the Beer–Lambert law the absorbance of a particular substance in a solution is directly proportional to its concentration; hence, the absorption spectroscopy can be used for a quantitative analysis. Also the absorptivity of a molecule is a wavelength-dependent parameter and its intensity depends on the chemical nature. Therefore the technique is an excellent analytical tool for the characterization and evaluation of many materials including

dental biomaterials and composites (Ikemura et al., 2008; Ikemura et al., 2010; Ikemura and Endo, 2010).

7.2.5 Nuclear magnetic resonance spectroscopy

NMR spectroscopy provides a nondestructive method for the research on chemical structures, conformations, and dynamics of biomolecules. In contrast to the previously discussed spectroscopy techniques, NMR probes the nuclei of atoms and not the electrons. A spin-nuclei mechanism occurs in NMR that is often based on the behavior of specific atoms (1H, 13C, 15N, 31P, 19F) exposed to an external magnetic field. The atomic nuclei with nonzero spins are NMR active in the magnetic field and generate two low and high energy spin states with the energy difference of ΔE.

The schematic diagram of the NMR technique is shown in Fig. 7.2. An external magnetic field, which is often very strong in the range of 1−20 T for modern NMR instruments, is used to induce the energy difference ΔE between the two spin states of the nuclei. The sample is simultaneously exposed to a radio frequency (RF) transmitter. Depending on the magnitude of the ΔE, different wavelength of the RF field is absorbed, which is detected by an RF receiving circuit. The ΔE is a function of the external magnetic field as well as the characteristics of the specific atoms (1H, 13C, 15N, 31P, 19F) exposed to the external magnetic field. The NMR spectrum is collected either by sweeping the magnetic field while the frequency of the RF radiation id fixed, or by varying the frequency of the RF radiation under fixed magnetic field.

An NMR spectrum is exhibited as plot of the signal intensity (from the RF receiver) against chemical shift. The chemical shift can be described as the ratio of the difference between the frequencies of a given sample and that of a reference

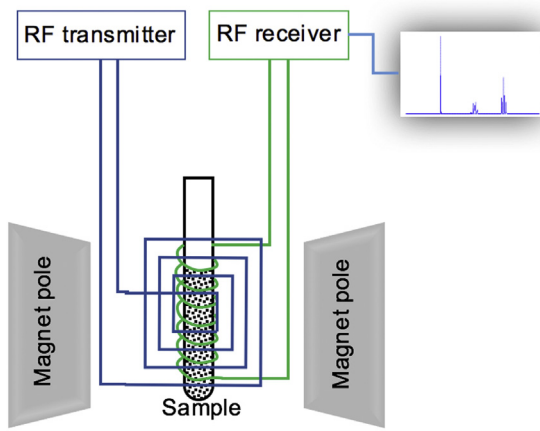

Figure 7.2 Schematic diagram of an NMR spectrometer.

compound, expressed in parts per million (ppm). The chemical shifts can reveal information about the covalent and noncovalent structure of the molecules.

NMR has also been applied in molecular level understanding of the mechanisms underpinning biomineralization, such as the onesused for bone repair and hard tissue regeneration of the teeth or in restorative dentistry (Goobes et al., 2007; Duer, 2015; Shetty and Kundabala, 2013).

7.2.6 Mass spectrometry

MS is a highly sensitive analytical technique which has been increasingly employed to characterize biomaterials based on the mass-to-charge ratio (m/z) of the gas-phase ions. The types of elements in the biomaterials and differences between the masses of the isotopic forms of a given atom can be determined by MS. A MS ionizes the material in the ionization source and employs mass-to-charge ratio (m/z) to separate the resulting ions in the mass analyzer. The ions are then detected, with or without fragmentation per their relative abundance.

The mass spectroscopy in conjunction with the chromatographic separation methods, including gas or liquid chromatography, has been often applied to characterize complex samples (Vékey et al., 2011).

Studies indicate that secondary ion mass spectrometry (SIMS) is a sensitive technique to characterize the biomaterials and biomineralized bone and dental tissues. Furthermore, the development of time-of-flight SIMS (TOF-SIMS) has also enhanced the mass spectroscopy sensitivity (Kanazawa et al., 2012; Ducheyne et al., 2015).

7.3 Physical characterizations techniques

7.3.1 Scanning electron microscope

Scanning electron microscope (SEM) is one of the common methods for imaging the microstructure and morphology of the materials. In SEM, an electron beam with low energy is radiated to the material and scans the surface of the sample. Several different interactions occur as the beam reaches and enters the material, which lead to the emission of photons and electrons from or near the sample surface (Sampath Kumar, 2013). In order to form an image the receiving signals produced from the electron−sample interactions are detected with different types of detectors depending on the mode of SEM being used. Different modes of SEM exist for characterization of materials (including biomaterials) such as the X-ray mapping, secondary electrons imaging, backscattered electrons imaging, electron channeling, and Auger electron microscopy (Sampath Kumar, 2013). A typical SEM consists of several components such as:

- The electron gun which is located on top of the column and emits electrons. The electrons are then accelerated to energy levels of typically 0.1−30 keV.

- Hairpin tungsten gun which makes a high diameter electron beam to form high-resolution images.
- Electromagnetic lenses and apertures which focus and shape the electron beam to form a small concentrated electron spot on the sample.
- A high-vacuum environment that allows electrons moving without being scattered or absorbed by the air.

7.3.2 Transmission electron microscope

Transmission electron microscopy (TEM) is often used to acquire direct information about the morphology, crystal structural, and composition of biomaterials to higher resolution than what is usually achievable with SEM. In this method, electrons are emitted from an electron gun located in a vacuum chamber and directed by electrostatic lenses onto the specimen (Sampath Kumar, 2013). Due to the interactions of the electrons with the sample, depending on the sample density, some electrons undergo scattering (or absorption), and some others pass through the sample. The sample must be thin enough so that the number of the transmitted electrons is sufficient for imaging. These electrons pass through the sample and hit the detector, such as a florescent screen, at the bottom of the microscope and an image. The image intensity follows the sample density. The denser the sample, the less electron pass through it, and the darker image is generated (Agrawal et al., 2013).

Achieving a high-resolution image in nanometer or less scale is the most important advantage of TEM. A small electron wavelength can lead to very high resolution in the order of less than a nanometer. The TEM has also some limitations like sample damage (due to the electron beam energy), low contrast in low atomic materials, and small depth of resolution that results in two-dimensional images (Sampath Kumar, 2013).

In comparison with SEM, TEM sample preparation, equipment operation, and data interpretation are more complex and require more time and skills (Agrawal et al., 2013).

7.3.3 Atomic force microscope

Atomic force microscopy (AFM) is a technique to image and analyze almost all kinds of surfaces (hard or soft, insulated or conductor, synthetic or natural). The AFM image reveals the three-dimensional features of the surface with spatial resolutions of nanometer or angstrom scale (Haugstad, 2012; Agrawal et al., 2013).

In AFM, a sharp tip is automatically dragged through the surface of the material and records its topographic image. The tip is attached to a flexible beam, and the force between the tip and the sample surface deflects the beam elastically. An optical system is used to record the beam deflection, which is proportional to the interatomic forces (Sampath Kumar, 2013).

Different modes of AFM have been developed based on the tip−surface interactions, as shown in Fig. 7.3:

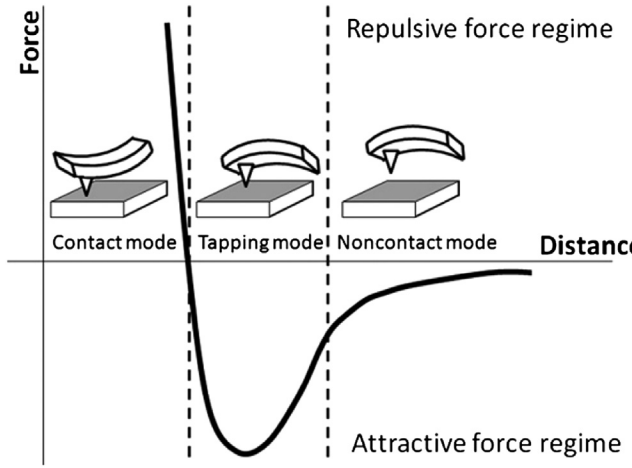

Figure 7.3 Schematic diagram of the different modes of AFM and the force versus distance for each mode (Sampath Kumar, 2013).

- Dynamic force or tapping mode, in which the tip oscillates and moves near the surface of the sample. The oscillations lead to periodic contact of the tip with the surface.
- Contact mode, in which the tip moves over the surface of the sample and experiences a strong repulsive force that bends the beam.
- Noncontact mode, in which the tip moves close to the surface (farther than the case of the dynamic mode and not touching the sample). The interaction forces in this mode are very low in the range of piconewtons. The noncontact mode does not damage the surface of the soft samples, which can happen in other modes due to the penetration of the probe into the sample (Sampath Kumar, 2013).

7.3.4 X-ray diffraction

X-ray diffraction (XRD) is a strong technique for the characterization of both the structure and composition of the materials. An X-ray diffractometer consists of an X-ray generator, a diffractometer which controls the direction of the X-ray beam and the position and orientation of the sample as well as the detector, a detector, and a computer to collect and analysis the data (Agrawal et al., 2013).

In this method, a monochromatic X-ray beam is radiated towards the material and the intensity of the diffracted beam is measured as a function of the angle of incident. The crystal interplanar space d, i.e., the distance between the atomic planes in the material, will be calculated as below:

$$2d = \sin\theta = \lambda$$

where λ is the wavelength of the X-ray used, and θ is the angle of incident. The interatomic plane spacing d and the diffraction angles reveal the material composition and structural features, such as shape and dimension of the unit cell, and the

width and shifts of the diffraction lines contain various information such as the crystallite sizes, residual stress, etc. (Sampath Kumar, 2013).

7.3.5 Contact angle measurement

The biocompatibility is one of the most important features of biomaterials that must be characterized carefully due the interactions with the tissues. Furthermore, the surface properties of biomaterials may also change due to exposure to the atmosphere or body environment. For example, this layers of oxide, carbon, or hydrocarbons may be formed on the surface of biomaterials even when they are exposed to normal atmosphere. Solid surfaces can be categorized in two groups of low- and high-energy surfaces. The first group has surface tension less than 100 dyn/cm, such as polymeric materials and organic compounds. In contrast, metallic and inorganic surfaces have surface tension of almost 200−500 dyn/cm and are placed in the second group (Sampath Kumar, 2013).

Quite often, special treatments are used to clean the surface of materials and change it into a hydrophilic surface. Contact angle measurement is one of the techniques to determine the hydrophilicity of the surfaces (Sampath Kumar, 2013). This measurement shows the physical properties of the surface or the coating such as adhesion, wettability, and cleanliness.

Wettability especially indicates the interaction between the liquid and the solid surface. Different techniques are used to measure this parameter, including direct optical methods and indirect force methods. Among these methods drop shape analysis and axisymmetric drop shape analysis are very common. The latter method is especially very accurate with a reproducibility of $\pm 2^{\circ}$ and is appropriate for contact angle measurement of ultra-small droplets (Yuan and Lee, 2013).

The angle between the surface and the tangent line at the contact point of the drop with the surface is called the contact angle as shown in Fig. 7.4. The contact angle is a quantitative value that determines the hydrophilicity of the surface. The angle decreases as the surface become more hydrophilic (Sampath Kumar, 2013). The contact angle can also indicate the amount of homogeneity or heterogeneity of a sample surface (Agrawal et al., 2013).

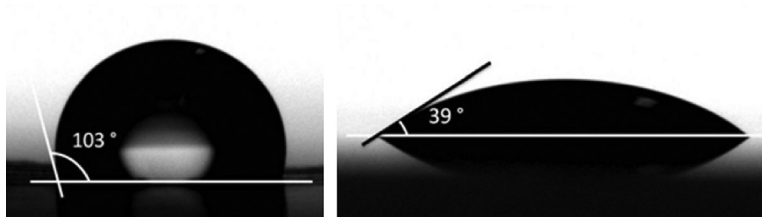

Figure 7.4 Contact angle image.

The spreading parameter, S, is a parameter that quantifies the wettability of a surface, and is defined as

$$S = \gamma_s - (\gamma_1 + \gamma_{s1})$$

when $S < 0$, liquid does not spread on the surface and the drop takes a spherical shape indicating partial or no wetting, whereas when $S > 0$, liquid spreads on the surface indicating a total wetting.

The contact angle can be measured in dynamic or static mode. The dynamic contact angle is produced during wetting, i.e., advancing angle or dewetting, i.e., receding angle. However, in the stats contact angle, the contact area between the surface and the liquid is not changed during the measurement. In the case of dynamic contact angle the advancing contact angle (θ_A) is simultaneously measured as the volume of the drop is increased on the surface while the receding contact angle (θ_R) is obtained when removing similar volume of liquid form the surface (dewetting). The advancing contact angle which occurs on wetting is usually larger than the receding angle which occurs on dewetting. The difference of the two angles, $\theta_A - \theta_R$, is the called hysteresis, which can be used to evaluate the roughness of the surface.

7.3.6 Mercury intrusion porosimetry

Porosity is described as the percentage of void space in a solid and is an important character of a biomaterial to permit cell migration and proliferation, cell nutrition, and vascularization for tissue regeneration. Additionally, a porous surface provides noteworthy mechanical stability by enhancing the mechanical interlocking between the inserted biomaterial in human body and the surrounding natural tissue. In recent years, porous biomaterials have been seriously researched and extensively used as scaffolds in tissue engineering/regeneration, as well as drug delivery system (Sampath Kumar, 2013). Although the minimum pore size needed to permit the in-growth of mineralized tissue is to the order of 50 μm, larger pore sizes can increase the depth of infiltration of mineralized tissues into the biomaterial; however, these larger pore sizes debilitate the mechanical characteristics. Smaller pore size creates larger surface area, causing higher adsorption of cell-inducing proteins. As the optimal pore size is reliant on the biomaterial employed and its application, it is very crucial to examine the porosity and pore size of biomaterials (Sampath Kumar, 2013; Karageorgiou and Kaplan, 2005; Park and Lakes, 2007).

Porous materials may have two vital pore types: open and closed pores. The closed pores are inside the bulk material and not reachable to outside fluids. The open pore is a channel that is connected to the surface of the material and reachable to fluids. The open pores are categorized into dead-end pores, which terminate inside the material, and interconnected/through pores, which make the passage of fluids feasible. Based on the pore diameter the International Union of Pure and Applied Chemistry (IUPAC) has categorized pores into three groups;

micropores ($D < 2$ nm), mesopores ($2\text{ nm} < D < 50$ nm), and macropores ($D > 50$ nm). Interconnected macropores are a crucial factor for scaffolds, as they provide the potential to enable tissue in-growth (Park and Lakes, 2007).

Mercury intrusion porosimetry (MIP) is widely used for measuring pore size and porosity. The principle is based on the moving of nonwetting liquid through the pores needing pressure (P), which is inversely proportional to the diameter (D) of the pores (assuming the pore is cylindrical) and directly proportional to the surface tension of the liquid (γ) and the angle of contact (θ) with the solid surface as described by the Washburn equation (Park and Lakes, 2007):

$$P = -4\gamma \cos \theta / D$$

Mercury is nonwetting to most solid biomaterials and thus, is best choice for intrusion porosimetry, also because it will not spontaneously penetrate pores by capillary action. For mercury-solid system (θ is usually taken as 140° and γ of the mercury is about 0.48 N/m), the applied pressure is inversely proportional to the size of the pores (i.e., only slight pressure being needed to intrude mercury into large macropores), whereas much more prominent pressures are needed to force the required equilibrated mercury into small pores. It also demonstrates that under given external pressure P, mercury can oppose passage into pores smaller than D, but cannot oppose passage into pores larger than D. Therefore for any pressure, it can be described which pore sizes have been occupied with mercury and which sizes have not. Recording the dynamic intrusion of mercury into a porous structure under rigorously controlled pressures allows the generation of pore size/volume distributions within the porous biomaterial (Sampath Kumar, 2013).

Regular MIP analysis includes putting the biomaterial specimen into a container and emptying the container to eliminate contaminant gases and vapors. Subsequently, while still emptied, mercury is permitted to fill the container and then the pressure is enhanced towards encompassing, while recording the volume of mercury entering larger openings in the specimen. When ambient pressure has been reached, diameters approximately 12 mm have been filled with mercury. Afterward, the specimen container is placed in a pressure vessel and the volume of mercury that intrudes into the specimen due to enhancement in pressure is described by substituting pressure values into Washburn's equation. In order to have a volume sensitivity down to <1 μL a capillary tube is connected to the specimen cup, which serves both as the mercury reservoir during test and as an element of the mercury volume transducer, since just a small volume of mercury is needed to produce a long "string" in a little capillary. The combination of specimen cup and capillary stem is called a penetrometer. The principle source of mercury is eliminated subsequent to filling, and the pressure applied to the mercury in the capillary is transmitted from the far end of the capillary to the mercury encompassing the specimen in the specimen cup. The outer surface of the glass capillary stem is plated with metal to shape a coaxial capacitor alongside the mercury column in the stem. The length of the mercury column is the only variable in the value of the capacitance, and a little volume of mercury entering or leaving a small

capillary causes a huge change in length (and area) of the mercury column. Therefore capacitance calculations of the stem establish a very high determination sensitivity and resolution for the mercury volume passing into or out of the specimen cup for changes in the outside pressure. The graph of the applied pressures and the cumulative volumes of mercury intruded at each pressure is named the intrusion curve. Thus as pressure is decreased, mercury extrudes the pores and a graph of this process gives the extrusion curve. The intrusion and extrusion curves do not have the same path due to the shape of the pores and other physical phenomena. Notwithstanding, both the intrusion and extrusion curves provide data about the pore network in the analyzed biomaterial. Finally, it is worth mentioning that MIP analysis establishes a border pore size distribution more precisely and faster than other techniques (Sampath Kumar, 2013).

7.3.7 Gas adsorption measurements

The surface area, particle size, and porous structure of biomaterials can be commonly examined by the determination of the amount of adsorbed inert gas such as nitrogen, argon, krypton, or carbon dioxide on solid surfaces. The gas adsorption can be categorized as either physical or chemical sorption. The physical adsorption of a gas includes weak molecular forces, such as van der Waals forces, whereas formation of a chemical bond establishes the driving force in chemical sorption. The physically adsorbed gases can be effortlessly eliminated by decreasing the partial pressure, but the chemically sorbed gases are generally difficult to eliminate from the solid surface. The physical sorption is employed not only to calculate surface area, but also to probe the entire surface, including irregularities and pore interiors. The equilibrium relation between the amounts of adsorbed gas with the partial pressure of the gas at constant temperature is called gas adsorption isotherm. In order to calculate the surface area and for pore analysis, the Brunauer, Emmett, and Teller adsorption isotherm is typically utilized, which includes adsorption of a monolayer of nitrogen gas molecules onto the surface of the powder by cooling and then heating to vaporize (desorption) the monolayer to calculate the amount of nitrogen adsorbed. The surface area is calculated from adsorption measurements by assuming spherical shape and unimodal distribution of the nanoparticles, and the surface area can then be simply converted into particle size. It is important to point out that, before calculating, the specimen is heat-treated in a vacuum or flowing gas to eliminate any contaminants (Sampath Kumar, 2013; Park and Lakes, 2007).

7.4 Biological characterization technique

Biocompatibility of biomaterials can be assessed using the in vitro and in vivo tests. In vitro tests are performed outside of the body, often employing cell culture systems and the aim is to simulate the clinical situation. In vivo tests are performed

Table 7.1 Advantage and disadvantage of in vivo and in vitro test

In vitro tests	In vivo tests
Advantage	Advantage
Experimentally controllable	Simulate real body condition
Repeatable	Clinically relevant
Fast	
Inexpensive	
Simple	
Small amount of test material is required.	
Decreasing the use of animal	
Have a quality control	
Range of applications is vast	
Disadvantage	Disadvantage
Chronic effects cannot be tested	Expensive
	Time consuming
	Ethical and regulatory issues

Table 7.2 Recommended standards by ISO, USP, and ASTM

ISO	USP	ASTM
Cytotoxicity	Cytotoxicity	Cell culture cytotoxicity
Sensitization	Sensitization	Sensitization
Genotoxicity	Genotoxicity	Skin irritation
Implantation	Implantation	Systemic toxicity
Chronic toxicity	Chronic toxicity	Short-term implantation
Carcinogenicity	Carcinogenicity	Long-term implantation
	Irritation	Genotoxicity
	Systemic toxicity (acute)	Carcinogenicity
	Subchronic toxicity	

inside the body of the living organism and are more clinically relevant than the in vitro tests. In vitro tests are frequently carried out as screening tests prior to in vivo assessment as the in vitro tests are simpler and cheaper than the in vivo tests and there are ethical issues associated with the use of animals for research. The in vivo and in vitro tests both have advantages and limitations. Table 7.1 displays the advantages and disadvantages of in vivo and in vitro tests (Cogliano et al., 2012; Di Silvio, 2008; Hanks et al., 1996).

A summary of the biocompatibility tests as recommended by the American Society for Testing Materials (ASTM), the United States Pharmacopeia (USP), and the International Organization for Standardization (ISO) for implantable devices and biomaterials is provided in Table 7.2 (Koschwanez and Reichert, 2007).

7.4.1 In vitro characterization

In vitro test systems often include three main components: a biological system, cell/material contact, and a biological end-point (Moharamzadeh et al., 2009). Biological system can be either 2D or 3D cell cultures or a whole organ culture. Cell/material contact can be established in three different ways: direct contact, indirect contact such as Agar overlay technique, and contact through extracts and eluates. Various biological endpoints can be assessed to record the response of the biological system to the tested materials.

7.4.1.1 Cytotoxicity testing

Cytotoxicity assay is a test for analyzing the cytotoxic effects of the material and medical device on the living organism (Rosengren et al., 2005). It was the earliest and simplest in vitro technique that was designed for biocompatibility evaluation of materials. Examples of biological endpoints used in cytotoxicity testing include:

- Morphological assessment using histology or ultrastructural analysis of the cells using the SEM or TEM.
- Cell viability and proliferation assays such as the Alamar blue assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, neutral red uptake, propidium iodide assay, lactate dehydrogenase assay, bromodeoxyuridine incorporation assay, 3H-thymidine incorporation assay, and DNA or protein content measurement.
- Cell function assays such as the measurement of the release of inflammatory markers, glutathione determination, heat-shock protein assay and apoptosis assay (Zhang et al., 2009; Camps and About, 2003; Seifalian et al.).

7.4.1.2 Hemocompatibility testing

Hemocompatibility testing is a method for studying of adverse effects (e.g., thrombosis, hemolysis, activation of platelets, and complement pathway) and interaction between the blood and the material or the medical device (Ratner et al., 2012). One of the main issues about hemocompatibility is inadequate standards for anticoagulation. Therefore it can be difficult to classify the material as hemocompatible or non-hemocompatible (Braune et al., 2013). The important aspects in hemocompatibility testing include physical and chemical characteristics, stability of the materials, test conditions, controls, reference materials, and plausibility aspects.

7.4.1.3 Genotoxicity and carcinogenicity testing

Genotoxicity and carcinogenicity testing are for studying the genotoxic effect (e.g., gene mutation, change in DNA, and chromosomal alterations) and the carcinogen effects of biomaterial/implant device on the living system (Ratner et al., 2012; Landsiedel et al., 2009). Genotoxic effects can be direct (DNA target) or indirect (non-DNA target) (Graziano and Jacobson-Kram, 2015). Genotoxic effects of nanomaterials have been evaluated by micronucleus assay, comet assay, and Ames test (bacterial reversed mutation assay) (Landsiedel et al., 2009). Ames test is a method

for evaluating mutagenic effects of implant device, chemicals, and drug utilizing bacteria to detect carcinogens and mutagens. The advantage of Ames test is its simplicity, low cost and being a fast assay (Seifalian et al.). Comet assay is another test to detect early DNA damage and it is a fast, sensitive, and simple technique (Kim et al., 2013; Seifalian et al.).

7.4.1.4 Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive in vitro method and has a crucial role in medical science and biomaterial fields. RT-PCR is used for detecting and comparing the levels of mRNA and the surface proteins (Leong et al., 2007; Wang and Brown, 1999). PCR can be performed in real-time PCR and end-point PCR. Relative and absolute quantification (aqPCR) are two models of real-time RT-PCR. The main role of end-point PCR in biomaterial fields is the measurement of gene expression. The absolute quantification is preferred to end-point PCR for detecting levels of gene expression but end-point PCR is usually used because it has low cost and needs simple equipment (Mauney et al., 2005).

7.4.2 In vivo characterization

In vivo testing of biomaterials and medical devices include implantation testing, sensitization testing, irritation testing, and toxicity tests (Di Silvio, 2008).

7.4.2.1 Sensitization, irritation, and toxicity tests

The innate immune system and adaptive immune system have important roles in immune responses (Di Silvio, 2008). Sensitization is an increase in immune response and acute or delayed hypersensitivity to a biomaterial in living system (Overmier, 2002) where utilization of the material can result in irritant effects and localized inflammatory reaction on skin (Robinson et al., 1990b). Sensitization testing can be time consuming as it requires a film of material/chemical agent in saline solution to be placed on the skin of the test subject and the symptoms of adverse effect of biomaterial to be monitored over time (Dumitriu and Popa, 2013). Guinea Pig Maximization, Buehler, and Murine Local Lymph Node Assay are examples of sensitization testing (Robinson et al., 1990a; Kimber et al., 2001). Intracutaneous reactivity, materials-mediated pyrogen test, acute systemic toxicity, subacute systemic toxicity, subchronic systemic toxicity, and chronic toxicity tests are examples of other in vivo tests that are often performed on animals to assess the irritation and toxicity of the materials (Ratner et al., 2012).

7.4.2.2 Implantation testing

In implantation tests, materials are implanted into the connective tissue, muscle, or into the bone of an animal to assess the adverse and pathological effects of products and materials on the function and structure of the tissues (Ratner et al., 2012). The pathological effects can be analyzed at gross level or at microscopic level using

histological techniques to demonstrate tissue necrosis and apoptosis, cell proliferation, thrombus formation, collagen deposition, and endothelization (Seifalian et al.). Short-term and long-term testing can be used to assess the immediate and delayed tissue responses to the implanted materials.

7.4.2.3 Biodegradation test

Some degradable biomaterials can leach out degradation products (e.g., impurities, catalysts, and corrosion products) to the adjacent tissues and distant organs. Biodegradation of materials occur through hydrolytic mechanisms. In vivo biodegradation tests have an important role for studying of effects biodegradation of material and medical device in living tissue. Histological analysis can identify biological and tissue responses in the living system (Park and Bronzino, 2002; Seifalian et al.; Ratner et al., 2012).

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