3 Inorganic Biomaterials Characterisation

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3.1 Introduction

The biocompatibility of a material is crucial in branding it as a biomaterial. Building on the previous biocompatibility chapter, this chapter mainly focuses on the assessment of biocompatibility. The main aims of biocompatibility assessment are: 1) raw material characterisation; 2) *in vitro*; and 3) *in vivo* assessment of materials. **Figure 3.1** shows a schematic representation of the components of biocompatibility assessment. This chapter will deal with each of these characterisations and assessment methods one by one in the following sections.

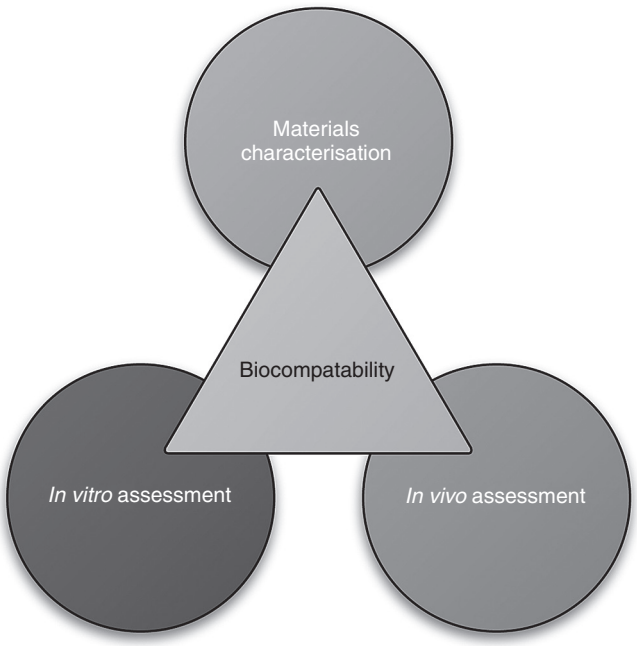

Figure 3.1 Major aspects of biocompatibility assessment

3.2 Raw Materials Characterisation

3.2.1 Introduction

Biomaterials have distinct bulk and surface characteristics. Knowledge of these characteristics is important in selecting the appropriate material for biomedical applications. An understanding of the methods used to characterise biomaterials is therefore essential for anyone working within the field of biomaterials engineering. Biomaterials characterisation deals mainly with the chemical, physical and mechanical characterisation of bulk and surface properties. This section hopes to provide a clear and concise guide to help biomaterials researchers select the appropriate tool from the vast array of specialised techniques available to them. Please see **Table 3.1** for a summary of the discussed methods.

3.2.2 What are Bulk and Surface Properties?

The properties of a material are not homogeneous but differ depending on a number of factors. Bulk properties are defined as those that remain constant regardless of the amount of material. For the purposes of biomaterials, the bulk can be seen as everything that is not part of the surface. On the other hand, the surface of a material is the termination of the three-dimensional (3D) bulk [1]. This area of the material represents an increase in energy and here dangling or unsaturated bonds can be found. These dangling bonds form new bonds when within a reactive environment, serving to lower the surface energy. It is therefore often the case that the properties of the surface differ significantly from that of the bulk. A practical example of these principles at work is the composition of a titanium orthopaedic implant. Within a fraction of second of exposure to an oxygen-rich environment, a 2–10 nm surface layer of titanium dioxide (TiO_2) builds up (**Figure 3.2**). Various other environmental contaminants are also adsorbed onto the surface, including hydrocarbons. Thus, the sum of the bulk and surface properties determine, to a large extent, the successful application of any particular biomaterial. Special emphasis is often placed on surface properties as this contributes directly to the *in vitro* response to the biomaterial including protein adsorption, cell adhesion, cell growth and blood compatibility. For this reason, specialised techniques are required to adequately characterise the surface of a biomaterial [2].

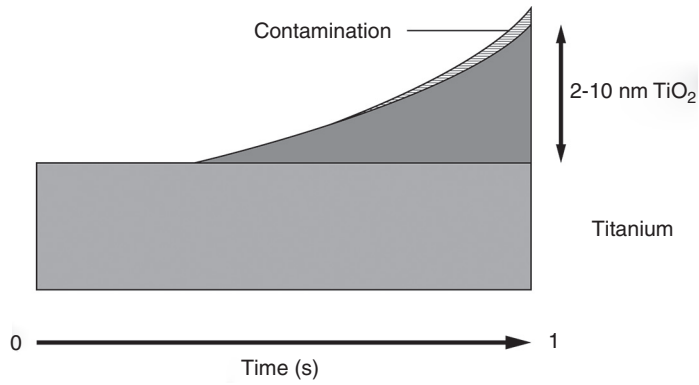

Figure 3.2 Dynamic changes at the interface of a titanium implant when exposed to air

3.2.3 Bulk Characterisation of Biomaterials

3.2.3.1 Transmission Electron Microscopy

- Application

  Transmission electron microscopy (TEM) is used to study the internal structure of biomaterials at a high resolution and is able to provide morphological, crystallographic and compositional information of inorganic materials. TEM allows imaging of materials to resolutions in the order of a fraction of a nanometre while simultaneously obtaining diffraction information. Use of high energy electrons (>100 kV) results in the excitation of atoms in the specimen allowing further spectroscopic techniques, including energy-dispersive X-ray spectroscopy (EDS), to provide chemical characterisation [3].

- Theory

  The transmission electron microscope is the original form of the electron microscope. In its current form it is one of the most powerful techniques available to the biomaterials researcher. During TEM, a beam of electrons is produced by an electron gun and then focused by a series of electrostatic lenses. This focused beam illuminates a thin specimen mounted on a grid. As the electron beam passes through the specimen, some electrons are transmitted whilst others are scattered or refracted. The typical accelerating voltage (the voltage used to drive the electron beam) is in the order of 100 kV to 1 MV, which allows penetration of samples <50 nm thick. Finally, the transmitted electrons are displayed using a fluorescent screen.

- Advantages

  - Very high resolution (<10 Å).
  - Provides information on the chemical structure.

- Disadvantages

  - Sample preparation is time-consuming.
  - Equipment is large and expensive.
  - TEM is limited to samples that can tolerate a vacuum and are electron transparent.

- Example

  Analysis of the particle size of carbonate anion intercalated magnesium-aluminium-layered double hydroxide and other inorganic materials such as hydroxyapatite (HA), quantum dots (QD) and ferrous nanoparticles.

3.2.3.2 X-ray Diffraction Spectroscopy

- Application

  X-ray diffraction (XRD) spectroscopy is a nondestructive analytical technique which characterises the crystalline phases and orientation of raw materials based on the arrangement of the atoms in their crystal structures. The crystal structure of a material consists of different layers or planes with a similar function to a semitransparent mirror. XRD uses a collection of single-phase diffraction patterns whose repeat distance is similar to the distances between the phases of the crystal structure. As the wavelength of X-rays is applied to the material it reflects at about the same angle of reflection as that of the phases of the crystal structure, leading to the phenomenon of diffraction. XRD spectroscopy is a very useful method to determine the structural properties (e.g., strain, grain size, phase composition and thermal expansion) of a raw material. It can also be used to measure the thickness of thin films and multilayers within a structure. Furthermore, it can be used to measure the size, shape and internal stress of small crystalline regions within the material [4].

- Theory

  The phenomenon of diffraction is based on Bragg's law, which provides the angles for coherent and incoherent scattering from the crystal structure of the material. According to Bragg's law, diffraction takes place when $2dsin\theta = n\lambda$, where n is

an integer, λ is the wavelength of the incident wave, d is the spacing between the planes in the atomic lattice, and θ is the angle between the incident ray and the scattering planes.

When X-ray beams are directed at the material they become scattered and create peaks of scattered intensity where the following two conditions apply:

- The angle of incidence is equal to the angle of scattering.
- The path length difference is equal to an integer number of wavelengths. The pattern of XRD is unique to each substance, which allows the identification of phases within a given sample.

- Advantages

  - Nondestructive method.
  - Powerful and rapid technique.
  - Minimal sample preparation required.
  - Data interpretation is relatively simple.
  - Can be used to identify unknown materials by comparing their crystal structure to the library of the materials.

- Disadvantages

  - Specimen displacement.
  - Instrument misalignment.
  - Peak overlay may occur.
  - Has size limitations (more accurate on larger crystalline structures).
  - For mixed materials, the detection limit is ~2% of the sample.

3.2.3.3 Fourier-Transform Infrared Spectroscopy

- Application

  Fourier-transform infrared spectroscopy (FTIR) is the most common form of infrared spectroscopy. It is a form of absorption spectroscopy which results in a characteristic 'signature' absorption spectrum. This absorption spectrum allows the quantitative identification of many inorganic compounds, determination of the molecular composition of surfaces and determination of molecular orientation [5].

- Theory

  Infrared spectroscopy works *via* measuring the absorption of electromagnetic radiation (EM) with frequencies between 12,800 cm^{-1} and 10 cm^{-1}. When exposed to infrared radiation, vibrational excitation is induced in covalently bonded atoms of the sample causing a change in dipole moment. These absorptions result in absorption peaks in the infrared (IR) spectrum. Absorption peaks are presented as wavenumbers (ν) with the unit cm^{-1}. This represents the number of waves per centimetre and is used as it is proportional to energy. The IR spectrum is subdivided into near-IR (4,000–14,000 cm^{-1}), mid-IR (400–4,000 cm^{-1}) and far IR (25–400 cm^{-1}). Mid-IR is commonly used to characterise materials as this region corresponds to the majority of primary absorption frequencies. For inorganic materials, far-IR is sometimes used as inorganic compounds such as silicides, borides and nitrides do not absorb radiation in the 4,000–400 cm^{-1} range.

FTIR differs from dispersive IR spectrometers through the use of a Fourier-Transform applied to an interferogram. The main benefit derived is the ability to measure multiple frequencies of IR radiation simultaneously compared with the more time-consuming method of measuring one frequency at a time.

- Advantages

  - Very fast characterisation of biomaterials as all frequencies are measured simultaneously.
  - Nondestructive method.
  - Good signal-to-noise ratio.
  - Ability to characterise materials in solid, liquid and gaseous phases.
  - Accurate wavelength calibration allowing spectral subtraction to remove, for example, the spectrum of a solvent.

- Disadvantages

  - Cannot detect atoms or monoatomic ions as they don't contain any bonds.
  - Aqueous solutions are difficult to analyse as water absorbs IR.

3.2.3.4 Dynamic Light Scattering

- Application

  Dynamic light scattering (DLS) is a method of measuring the size, size distribution and shape of nanoparticles in solution. DLS is a versatile tool that can be used in

most situations where inorganic particles are in solution, one example being the measurement of QD size [6].

- Theory

  DLS works through the measurement of the intensity of light scattered by the molecules in the sample solution as a function of time. Due to Brownian motion in the solution, interference occurs in the scattered light. This causes a change in light intensity which is measured in relation to time. The faster the rate of diffusion or Brownian motion within the solution, the faster the intensity fluctuations will occur. Considering all other variables are controlled, the speed of diffusion is a result of the size of the particles. This allows the size or hydrodynamic radius of the particle to be inferred based on the rate of diffusion.

- Advantages

  - Fast characterisation of particle size in solution.
  - Nondestructive method.
  - Automated and not dependent on an operator.
  - Can be performed on a small sample.

- Disadvantages

  - Sample can be easily contaminated.
  - Sensitive to mechanical disturbances.
  - Limited to measuring particles in solution.

3.2.3.5 Mercury Intrusion Porosimetry

- Application

  Porosity is an important property of many biomaterials and is defined as the ratio of void space to the total volume. Porous and connected networks are essential for cell proliferation, nutrition, cell migration, vascularisation and new tissue formation. Furthermore, a porous material allows better biointegration through an increased surface area and therefore larger biointerface. Mercury intrusion porosimetry (MIP) allows the biomaterials researcher to measure total pore diameter and mean pore size.

- Theory

  During MIP, the sample is placed in a mercury penetrometer and is subsequently infused with mercury using continuously increasing pressures. The method is based on the principle that the pressure required to fill the pores of a sample, with a nonwetting liquid, is inversely proportional to the diameter of the pores. Thus, higher pressures will be required to fill pores of smaller diameters due to higher surface tension forces. Although most nonwetting liquids can be used, mercury is especially suitable as it does not fill pores by capillary action. By monitoring the intrusion of mercury into the pores under highly controlled pressures, the pore size and volume distribution can be calculated.

  Although MIP is useful for the generalised characterisation of porosity, it has drawbacks. Regarding the overall porosity, MIP does not take into account the existence of closed pores. Furthermore, it cannot differentiate between blind and through pores; this is important as through pores have been shown to positively impact the performance of biomaterials. In addition, the shape and morphology of pores cannot be measured. The results garnered are therefore more an approximation and rather crude. Practical aspects also hamper the overall usefulness of the technique as materials that cannot withstand the required pressures can be destroyed, nor can easily compressible materials be measured.

- Advantages

  - Almost any material can be measured.
  - Can characterise a large range of mean pore sizes.

- Disadvantages

  - Pore morphology cannot be accurately measured.
  - Only measures mean pore size.
  - Some materials cannot withstand high pressure environments.
  - Does not measure closed pores.
  - Destructive due to the toxicity of mercury.

3.2.3.6 X-ray Computed Tomography

- Application

  X-ray computed tomography (CT) imaging can be used to obtain accurate 3D morphological data on scaffolds. In particular, CT imaging overcomes the

disadvantages of MIP by providing detailed data on porosity, pore sizes, closed pores and pore interconnectedness.

- Theory

  CT imaging works by dividing the sample into a series of two-dimensional (2D) X-ray slices. Once the entire sample has been sliced, 3D modelling software builds an intricate 3D model of the sample from the individual 2D slices. 3D computation can then be performed on the model to calculate accurate data on the required properties. This technique is applicable to most bioceramics and bioglasses, but optimal results are difficult when dealing with metals due to heavily attenuated X-rays.

- Advantages

  - Provides an intricate 3D model of exterior and interior surfaces.
  - Accurate porosity measurements.
  - Nondestructive technique.

- Disadvantages

  - Cannot be used on metallic materials.

3.2.4 Surface Characterisation of Biomaterials

3.2.4.1 X-ray Photoelectron Spectroscopy

- Application

  X-ray photoelectron spectroscopy (XPS), also called electron spectroscopy, for chemical analysis is a surface characterisation method able to sample to a depth of 2–5 nm. XPS provides quantitative and qualitative information about the elements present at the surface and the bonds between them. XPS is unable to detect hydrogen and helium. XPS is useful for determining elemental composition, chemical state information and surface imaging.

- Theory

  XPS is based on the photoelectric effect, described by Albert Einstein in 1905. During XPS a sample is placed in an ultrahigh vacuum chamber and then bombarded with X-ray radiation. This causes a transfer of energy from the X-rays

and results in the emission of a photoelectron. The kinetic energy of the emitted electron is calculated using the binding energy of the electron and the energy of the incident X-ray. The kinetic energy is characteristic of a particular element and is then plotted on a graph of number of electrons detected (intensity – Y-axis) over binding energy (electron volts – X-axis).

- Advantages

  - Can characterise all elements other than hydrogen and helium.
  - Can provide information on the chemical states of samples.
  - XPS can differentiate between the oxidation states of molecules.
  - Simple to interpret and widely used.

- Disadvantages

  - Poor absolute sensitivity with minimum levels of 0.1–0.5% required for detection.

3.2.4.2 Secondary Ion Mass Spectrometry

- Application

  Secondary ion mass spectrometry (SIMS) is an important characterisation tool and compliments the use of XPS. SIMS produces a mass spectrum of atoms and molecules, and is able to sample to a depth of around 1 nm. SIMS is able to identify all elements, suggest the molecular structure and is particularly suitable for inorganic characterisation. It has also been used for submicron imaging and chemical mapping of both organic and inorganic materials.

- Theory

  Similarly to XPS, SIMS takes place within an ultrahigh vacuum chamber. SIMS analysis uses a focused and accelerated beam of ions (5–20 keV) to bombard the sample. This bombardment causes the sputtering of the surface particles into the vacuum. These ejected particles are secondary ions, radicals and neutral particles. The ejected secondary ions are then measured using mass spectrometry. The ratio of the mass-to-charge (m/z) of the ejected ions is measured and used to characterise the particles. This provides a spectrum of particle count over mass-to-charge (m/z) ratio. Typical measurements are made using a time-of-flight mass analyser.

  Two types of SIMS analysis exist, known as dynamic SIMS and static SIMS. Static SIMS aims to minimise surface destruction through the use of an adjusted

ion dose, resulting in minimal ion sputtering. By limiting the incident ion dose, sputtering will be limited to the outermost monolayer, essentially leaving the surface unchanged.

Dynamic SIMS is a more complex technique which yields more information but is destructive in nature. During dynamic SIMS, relatively high ion doses are used to maximise secondary ion sputtering. Enough material is sputtered that the surface appreciably erodes over time and this provides a spectrum which will be representative of the average composition of the sampled volume. This therefore makes dynamic SIMS a useful technique for depth profiling of the surface by measuring the continuous peak intensity as a function of time.

- Advantages

  - Very high surface specificity (higher than XPS).
  - Can detect hydrogen and helium.
  - Can generate a wealth of molecular and structural information.
  - Particularly suitable for inorganic applications.
  - Carbon buckyballs can be used as sputtering ions to provide a less destructive method of analysing sensitive biomaterial surfaces.

- Disadvantages

  - Quantification is difficult due to the poorly understood sputtering process.
  - Can be destructive; however, this is utilised by dynamic SIMS to allow depth profiling of biomaterials.

3.2.4.3 Optical Microscopy and Confocal Microscopy

- Application

  Optical microscopy is the most commonly used method to examine the microstructure of a biomaterial. Techniques developed by metallurgists to investigate metal surfaces have also been applied to other biomaterials, including ceramics. On its most basic level, light microscopy provides a visual image of the surface of a material and will allow measurements of surface characteristics within the limits of the resolution afforded by the wavelength of visible light [7].

- Theory

  There are many variations of optical microscopy with modifications and improvements in design for the purpose of increasing resolution, magnification

and contrast, and reduction in aberrations and artefacts. Here, the basic principles behind optical microscopes as well as a brief exploration of confocal microscopy will be introduced.

The main components of an optical microscope include the ocular lens, the objective lens, the specimen stage and the light source. Specimens are illuminated by the light source after which the light is focused and the image magnified by a series of lenses. The lens closest to the specimen is known as the objective lens and is responsible for the majority of the magnification. The image produced is further magnified and focused by the ocular or eyepiece lens. Optical microscopy is fundamentally limited to the resolution of the system. Resolution is defined as the minimum distance between two points at which they can still be told apart or *resolved* and is directly proportional to the wavelength of the radiation being used. In the case of optical microscopy, the best theoretical resolution possible is around 0.2 μm at a wavelength of 400 nm (violet light). Theoretically, the magnification of an image is limitless but in practice the inability to distinguish important features means that the *effective magnification* is limited to around 1,000x. Of equal importance to magnification is the contrast and brightness of the image. Brightness decreases as magnification increases and needs to be carefully adjusted to allow the best possible visibility of the specimen.

Confocal microscopy is a form of optical microscopy which utilises spatial filtering, allowing an improved resolution as well as 3D imaging. In traditional optical microscopy, high resolution images are often obscured by a haze. A confocal microscope is able to isolate the plane of focus and remove the obscuring haze by utilising a pinhole or iris diaphragm. The main limitation of this system is that only the small area of focus is visible at one time. Confocal microscopes overcome this by using moving mirrors to scan an image which is then stitched together by a computer. Furthermore, by compiling the 2D images a 3D image can be constructed.

- Advantages
    - Optical microscopes are cheaper in comparison to electron microscopes.
    - Direct viewing of samples with relatively simple preparation.
    - Samples can be viewed in colour.
    - Samples do not need to be viewed in a vacuum.
- Disadvantages
    - Low resolution compared with other imaging techniques.

3.2.4.4 Scanning Electron Microscopy

- Application

  Scanning electron microscopy (SEM) is able to provide very high resolution imaging of solid surfaces and is widely employed in the characterisation of biomaterials. SEM provides a large depth of field in comparison to its resolution and can analyse segments of 5 μm to 1 cm at a resolution of 50–100 nm. SEM is a versatile tool and can provide information on external morphology, chemical composition, crystalline structure and orientation of the surface of a material. It is useful to note that SEM is often combined with EDS to provide chemical characterisation of the sample surface. This will be explained in more detail below.

- Theory

  As with other forms of electron microscopy, an electron gun is used to produce a high energy beam of electrons which is subsequently focused by a series of EM lenses. The incident electrons produce a multitude of different signals as they collide and decelerate within the sample. These include secondary electrons, backscattered electrons, diffracted backscattered electrons, photons and others. SEM mostly utilises secondary electron detection to form an image of the sample and provides information regarding the morphology of the sample. Highlighting the versatility of SEM, the other resultant signals can also be measured to provide additional information. Commonly, backscattered electrons can be used to provide contact in multiphase samples. Also, as mentioned, EDS can characterise the chemical makeup of the material surface. This is possible due to the resultant X-rays, emitted as electrons within the sample atoms, returning to their lower energy state after inelastic collisions with the incident electrons.

- Advantages

  - SEM is the most versatile tool available to the biomaterials scientist.
  - High resolution images, especially when compared with optical microscopy.
  - SEM creates beautifully immersive images.
  - SEM can provide a wealth of information when combined with other techniques.

- Disadvantages

  - Samples must be coated in a conductive material for the majority of conventional SEM.
  - SEM generally requires a high vacuum although other models are available that allow low vacuum environments.
  - SEM chambers can be small, limiting the size of the sample.

3.2.4.5 Contact Angle Measurement

- Application

  Contact angle measurement is used to characterise the hydrophilicity/hydrophobicity of biomaterials. As mentioned earlier, these properties are essential to the future in vivo application of biomaterials and directly affects cell adhesion, migration and proliferation. There are a number of methods to measure contact angles, the most common of which will be discussed here [8].

- Theory

  The contact angle is the angle formed at the intersect between the liquid-solid and liquid-gas interface. This angle is determined by the thermodynamic properties of the two materials and gives an indication regarding the wettability of a material. When a liquid spreads out over a surface, a small contact angle is measured and conversely, a large contact angle is measured when the liquid is stable and forms a bead. A contact angle of less than 90^{o} indicates the material is wettable or hydrophilic in nature and the opposite is true for a contact angle over 90^{o}. Therefore, an angle of 0^{o} indicates very high hydrophilicity. Superhydrophobic surfaces, such as those seen on the lotus leaf, often have contact angles over 150^{o}. The thermodynamics governing contact angles is beyond the scope of this section. It is suffice to say that the most important point to understand in this case is the relationship between contact angle and wetting, and the applicability to bioengineering. Numerous methods exist to measure the contact angle, a few of which will be briefly discussed. Commonly used methods include the Wilhelmy method, the sessile drop method and the captive bubble method.

  The Wilhelmy method is a technique that indirectly measures the contact angle. The sample is attached to a balance, lowered and brought into contact with a liquid. The change in force detected by the balance represents the buoyancy and the wetting force. With a known liquid surface tension and perimeter of the solid, the contact angle can be calculated. The method is a dynamic one and can measure contact angle hysteresis at different wetting speeds. It is also accurate as it is not dependent on visual measurements.

  The sessile drop method is an optical method where a drop of liquid is placed on the solid surface and then measured by a contact angle goniometer. The method can be performed both statically and dynamically, where the dynamic version involves modifying the drop and measuring the advancing and receding angles. The sessile drop method is the most commonly used method in the field of bioengineering.

The captive bubble method involves measuring the contact angle by submerging the solid in the liquid and 'capturing' a bubble of gas. The air bubble is then measured using a digital camera and the contact angle manually calculated from the image.

3.2.5 Mechanical Characterisation of Biomaterials

3.2.5.1 Introduction

Detailed knowledge of the mechanical behaviour of biomaterials is essential for most applications. The importance is self-evident in many examples, such as dental implants, where the implant must be able to withstand the forces generated by mastication. Not only must a bioengineer be able to predict the failure of a prosthesis or implant, but also accurately predict the force relationships between the prosthesis and the body. For example, in the case where a prosthesis does not adequately transmit forces to the underlying tissue, such as bone, deficiencies in normal bone metabolism may lead to the prosthesis loosening and ultimately, failure. There have been many excellent books written on the topic of mechanical characterisation, so only commonly tested properties and corresponding techniques will be covered here [9, 10].

3.2.5.2 Tensile and Shear Properties

There are four forces that can be applied to a material; these are tensile, compressive, torsional and shear. In testing tensile strength, the properties measured are the engineering stress (σ) and engineering strain (ε). To measure these the sample is cut into a dog-bone shape and attached to a mechanical testing frame. This sample is then loaded over a longitudinal axis by moving one end of the frame. The force is applied, to a cross-sectional area of the sample, along the length of the sample and the stretched length of the sample is measured. By using these variables the engineering stress is calculated with the following formula:

$$\sigma = FA_0 \tag{3.1}$$

Where:
σ is the engineering stress.
F is the force applied.
A_0 is the original cross-sectional area of the sample.

The engineering strain is calculated as:

$$\varepsilon = l_i\text{-}l_0 FA_0 \tag{3.2}$$

Where:
ε is the engineering strain.
l_i is sample length.
l_0 is the original sample length.

Using the above formulae, a stress-strain graph can be plotted. Where the curve follows a linear relationship, stress is directly proportional to strain, a relationship known as Hooke's law. The slope of this curve gives the modulus of elasticity or stiffness of the material. Where Hooke's law applies, the deformation is elastic, meaning it can return to its original shape upon release of the load. For a high stiffness, a large stress is required for deformation to occur.

3.2.5.3 Time-dependant Properties

For biomaterials, short-term tests as mentioned previously do not paint the full picture. Longer term testing is necessary to predict the behaviour of biomaterials that may be expected to last for decades. One important time-dependent property to consider is creep.

- Creep

  Creep is the plastic deformation of a material under constant load over time. Creep is mostly applicable to polymers as it may occur at room temperature but can still occur in metals and ceramics. To test material creep, a constant tensile load is applied to a material whilst the temperature remains fixed. The strain is recorded as a function of time and plotted. Creep testing provides the steady creep rate of the material and the time to rupture for a given material at a given temperature.

Table 3.1 Raw material characterisation techniques

Technique	Surface or bulk	Information provided	Advantages	Disadvantages	Ceramics	Bioglass	Metals
TEM	Bulk	Morphological, crystallographic and chemical information	High resolution (<10 Å)	Sample preparation is difficult	✓	✓	✓
FTIR	Both	Chemical analysis, structural analysis and conductivity	Nondestructive. Very fast. Good signal-to-noise ratio	Cannot be used for monoatomic analysis	✓	✓	✓
DLS	Bulk	Provides size, size distribution and shape of nanoparticles in solution	Nondestructive. Fast. Can use very small sample sizes	Samples easily contaminated. Limited to particles in solution	✓	✓	✓
MIP	Bulk	Porosity. Mean pore diameter	Almost any porous material can be measured. Useful over a large range	Pore morphology not accurately characterised. Only measures mean pore size. Cannot measure closed pores	✓	✓	✓

Microcomputed tomography	Bulk	Detailed morphological data on pore size, porosity and pore interconnectedness	Accurate porosity measurements. Nondestructive	Cannot be performed on metals	✓	✓	✓
XPS	Surface	Elemental composition, chemical state and surface imaging	Characterises all elements besides helium and hydrogen. Provides information on chemical state and oxidation states	Poor absolute sensitivity	✓	✓	✓
SIMS	Surface	Elemental composition, molecular structure and depth analysis	Very high specificity (higher than XPS). Able to detect hydrogen and helium. Useful for inorganic applications	Difficult quantification. Dynamic SIMS is destructive	✓	✓	✓

3.2.6 Cytotoxicity Testing of Inorganic Biomaterials

There are two types of cytotoxicity test; qualitative and quantitative. Qualitative cytotoxicity tests, such as direct contact and agar diffusion, are good methods for screening purposes. However, the quantitative assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and tetrazolium salt (XTT) assays are preferred as they provide more data in a statistically comparable manner. In the cases of inorganic biomaterials such as bioglasses and bioceramics, MTT is the most commonly used assay. This is mainly because it can accurately quantify a low density cells and can be performed on either extracts or by direct contact. Another major advantage of this assay is that there is no need to subject the results to any interpretation by an analyst. Overall, it is a good assay for the fast screening of multiple samples with high accuracy. Despite various advantages leading to its frequent use, it should be noted that this particular assay does not differentiate between specific cellular death mechanisms, such as apoptosis or induced cell death, and in some cases fails to completely assess cellular damage as it only takes into account cellular death at its last stages.

In addition to the above assays, other *in vitro* tests such as alamar blue and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium can also be used to provide cell counts, based on colorimetric analysis, in order to measure the level of toxicity of inorganic biomaterials. These assays can be used on their own or in conjunction with other *in vitro* assays for the purposes of evaluation of the cytotoxicity of biomaterials (see **Table 3.2** and **Table 3.3**).

Table 3.2 Aspects of biocompatibility		
Aspects of biocompatibility	**Definition**	**Relevance to inorganic biomaterials**
Appropriate host response	The absence of toxicity, immunogenicity, carcinogenicity and thrombogenicity	Inorganic biomaterials must be able to perform with an appropriate host response, i.e., they should not cause any harm to the biological tissues of their host
Mechanical performance	A biomedical device or implant must perform in such a way as to not mechanically damage surrounding tissue	Example: Hydroxyapatite-based lumbar fusion cages that have sharp edges or those that are too stiff can lead to undesirable conditions, such as developing adjacent level spinal degeneration [11]
Cell-biomaterial interaction	The particular interactions (e.g., cell attachment/detachment, proliferation and differentiation) between host cells and an implanted biomaterial	This is an important aspect in regards to inorganic biomaterials. For example, a dental implant made of bioceramics requires attachment, proliferation and differentiation of cells to promote bone growth and to achieve integration between the natural bone and the bioceramic dental implant
Cytotoxicity	Biomaterial toxicity causing cell damage. Cytotoxicity can lead to inhibition of cell proliferation or cell death	Cytotoxicity due to inorganic biomaterials is usually due to chemical leaching from the material and this can adversely affect clinical outcomes
Genotoxicity	The toxic effect of biomaterials on the basepair sequence of the host genome	Inorganic biomaterials can cause genotoxicity depending on their type, concentration and the length of exposure. Currently, most of the developed inorganic biomaterials are considered nongenotoxic and are unlikely to cause any severe DNA damage. The development of inorganic radioactive nanoparticles for cancer therapy may raise questions regarding genotoxicity in the future

Mutagenicity	A material is said to be mutagenic if genotoxic damage fails to be repaired by cell repair mechanisms	Inorganic biomaterials are commonly incorporated with polymeric scaffolds seeded with stem cells for various medical applications. If these materials test positive for mutagenic properties it means that the biomaterial is not biocompatible as it can cause gene mutations and chromosomal aberrations on the stem cells incorporated in the polymeric scaffold leading to failure of bone repair or spinal fusion
Carcinogenicity	Refers to situations in which the alterations of DNA, as the result of the genotoxicity effects of a material, cause cells to grow uncontrollably and results in malignancy	Recently ceramic microspheres have been used to treat liver cancer [12]. Ceramic microspheres can be designed to attack cancer cells without affecting the normal cells however, this method is new and research scarce. It is still not clear as to what happens to the ceramic microsphere nano and microparticles as they degrade and if they might have any carcinogenic effect on the normal cells or encourage already present tumours to grow
Immunogenicity	The property of materials to facilitate an immune response in the human or animal body. Immunogenicity can be either wanted or unwanted	Inorganic materials can lead to high intensity of inflammatory/immune response if used at very high concentration or after exposure periods which are too long. However, unlike autograft or allograft materials, the risk of inflammatory/immune response is minimal
DNA: Deoxyribonucleic acid		

Table 3.3 Tests for *in vitro* assessment			
Test category	**Assay**	**Assay principles**	**Information obtained**
Cytotoxicity	**Qualitative**		
	Direct contact	The material under investigation is placed directly in contact with cells in a culture medium and then incubated, during which leachable chemicals from the biomaterial can diffuse into the culture medium and interact with the cell layer	The cell-material interaction can lead to malformation, degeneration and lysis of the cells, which are used a measure of the reactivity of the test sample
	Minimum essential medium elusion	An assay based on the actual use conditions of a test device, in which different extracting media and extraction condition conditions are used. Here, the test subject is placed onto an agar surface overlaid onto a monolayer of cells such as L929 cells from a mouse. A dye is used to identify the viable, stressed or lysed cells	Investigates the presence of toxic materials eluted from the test subject and presents this information in a semiquantitative measurement of cytotoxicity
	Agar fusion	Test sample is placed on the surface of a solidified agar made of cells covered with a solution of 2% agar and red dye. The agar containing the test sample is then incubated which allows live cells to take up the stain. Dead cells do not take up nor retain this dye	The toxicity of the test sample is measured by the amount of dye that has been lost from under and around the test sample using surface microscopy, allowing investigation of the material-cell interface

Cytotoxicity (continued)	Quantitative		
	MTT	Colorimetric assay that measures the reduction of the yellow, water-soluble MTT by mitochondrial succinate dehydrogenase, an enzyme complex	Only living cells are able to catalyse this biochemical reaction. Using a spectrophotometer, one can obtain a rapid quantification of the percentage of living cells in the test solution
	XTT	Water-soluble XTT is used instead of MTT, otherwise it's a similar principle. The water solubility of XTT allows the final solubilisation step to be avoided. XTT is a colourless/pale yellow chemical compound that turns light orange when reduced	The formasan product of XTT reduction is soluble and can be used in real-time assays to quantify the percentage of living cells in a solution
	Colony formation cytotoxicity	This assay measures the number colonies formed after exposing them to the material under investigation at different concentrations using the Chinese Hamster lung cell (v79) culture system. The formation of the colonies is measured (using a stereomicroscope) while cells are in their state of proliferation, as they are more sensitive to a toxic effect at this stage	The half maximal inhibitory concentration (IC50 value) is calculated to quantify the number of formed colonies. This assay provides information on every cell in the sample solution regarding its ability to undergo unlimited division, since only a fraction of seeded cells retains the capacity to produce colonies once in contact with a potentially cytotoxic testing material

Genotoxicity mutagenicity carcinogenicity	Ames	An effective reverse mutation assay to detect point mutations induced by the test material using several strains of the bacteria *Salmonella typhimurium*, which allows a quantifiable measurement of changes in phenotypic expression of their gene	This is a sensitive test to investigate the potential of a test material to react with DNA and alter its expression
	Single cell gel electrophoresis	Cells are encapsulated in a low-melting-point agarose suspension followed by lysis of cells in neutral or alkaline conditions. The suspended cell lysis is then subjected to electrophoresis. The extent of DNA damage is then determined by visual analysis with staining of DNA and calculating fluorescence	The assay measures if the test subject has the potential to cause a DNA strand beak in eukaryotic cells, in which the intensity of the comet (pattern of DNA migration) tail relative to the head reflects the number of DNA breaks
	Chromosomal aberration	Complementary to the Ames assay, it measures mutagenes and/or carcinogens of the test subject by quantifying and characterising the chromosomal aberration of mammalian cells	Allows investigation if the test subject has the potential to change the normal process of DNA replication and/or division of cells causing chromosomal aberration
	Mouse lymphona assay	Uses eukaryotic cell lines to detect point mutations and chromosome damage that result in a quantifiable change in phenotypic expression of a targeted gene	Determines the toxicity of the test subject by measuring the decrease in colony forming efficiency caused as the result of alterations in DNA expression

Bone remodelling	Alkaline phosphatase assay	Based on measuring ALP activity, a marker for osteogenic differentiation, by monitoring the colour change as the colourless *para*-nitrophenol phosphate is cleaved to *para*-nitrophenol and yellow phosphate	Determines if the test subject has the potential to change the production rate of phosphate, necessary for bone formation
	Calcium assay	Based on the o-cresolphthalein-calcium reaction in which a vivid purple o-cresolphthalein complex is formed which absorbs between 560 nm and 590 nm. It measures the total calcium concentration in biological test samples	Determines if the test material has any influence on the total calcium concentration, the main component of bones and teeth
	Osteocalcin ELISA	Measures protein levels of intact human osteocalcin in serum and plasma. Monoclonal antibodies, which target distinct epitopes of human osteocalcin, are used to measure the absorbance of the amount of substrate turnover, which is proportional to the concentration of osteocalcin	Determines if the test material has any influence on the total osteocalcin concentration, a high concentration component of natural bone
	Simulated body fluid	Effectively measures calcium phosphate (apatite) formation or precipitation on the surface of the test biomaterial	Determines the ability of the test material to form an apatite precipitation layer in SBF with ion concentrations almost the same as those of human blood plasma. This is a useful assay for predicting the *in vivo* bone bioactivity of an inorganic biomaterial

Degradation	Liquid chromatography	Pressurised liquid (a mixture of solvent, e.g., water and methanol) and a sample mixture are pumped into a column containing an active component, the sorbent, made of solid particles (e.g., silica) of up to 50 micrometres in size. The sorbent particles interact with different components of the sample mixture at different degrees, leading to their individual separation	Determines the individual components that make up the sample mixture as well as their quantity/ratio in respect to all the other components of that material. In the case of inorganic biomaterials, as they degrade, calcium is release into the biological system of the host. Liquid chromatography can be used to identify if calcium has been released; if yes, how much of it and at what rate
ALP: Alkaline phosphate ELISA: Enzyme-linked immunosorbent assay			

3.2.6.1 Genotoxicity, Mutagenicity and Carcinogenicity Testing of Inorganic Biomaterials

The Ames assay is a bacterial reversed mutation assay used worldwide as an initial evaluation of the mutagenic potential of new medical devices/implants, chemicals and drugs. Ames is a fast, simple, sensitive and economically justified assay which can be used for all inorganic biomaterials. Ames of inorganic biomaterials such as bioglasses and bioceramics are routinely carried out on tester *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537. It has been noticed from previous studies that some materials can be mutagenic to one tester strain while completely nontoxic to another. Therefore, it is best to use at least four strains to ensure the validity of the results [13].

The single cell gel electrophoresis assay, also known as the 'Comet assay', is another *in vitro* technique to evaluate DNA damage caused by inorganic biomaterials. The Comet assay is quick, uncomplicated, sensitive, accurate and cost-effective. What makes this test more interesting is that it can be applied to virtually any cell type and can be used alongside other standard assays, therefore, reducing the time and expenses associated with toxicity testing, while increasing the accuracy of information (**Tables 3.2** and **3.3**).

3.2.6.2 Other Useful In Vitro Assessment Tests Related to Inorganic Biomaterials

The medical application of inorganic biomaterials is well established in the literature. Most if not all of these studies have conducted some sort of *in vitro* biocompatibility tests. However, apart from toxicity and biocompatibility, *in vitro* studies can be used to investigate the success of such inorganic biomaterials in achieving their designed purpose, for example, bone tissue engineering.

HA, for example, has been extensively tested *in vitro* in order to investigate its interactions with living cells and tissues for the purpose of growing bone. In an ideal case, one would like a HA-based device or an implant seeded with stem cells, such as bone marrow-derived mesenchymal stem cells, to first interact with collagen, and later to observe the accumulation of proteins and cells on its surface followed by degradation of the material as the bone grows in its place. This process can be studied using *in vitro* assays such as quantitative DNA assays, alkaline phosphatase (ALP) assay, calcium assay and osteocalcin ELISA.

Quantitative *DNA assays* are generally used to determine the seeding efficiency and cell growth on a test subject. These assays are relatively simple, time and cost-effective

as well as accurate. The ALP assay, on the other hand, is based on measuring a membrane-bound enzyme, as a marker for osteogenic differentiation on the scaffolds. The ALP assay is a rapid and quantitative test which allows accurate determination of whether or not HA can be used to promote bone growth for various purposes, such as dental and orthopaedic applications. The *calcium assay* and *osteocalcin assay* tend to assess bone growth by measuring the amount of calcium or osteocalcin protein deposited in the cell-scaffold constructs, respectively. Calcium is a major material in bone and teeth mineralisation and hence is a good marker to assess bone growth.

All of the above assays are used to target a particular marker, for example, an enzyme or protein, to determine if bone growth will be of major use in clinical practice; however, it is not clear which assay has the better clinical application and the lack of or poor comparability between different assays makes it difficult to decide which of these tests should be definitively performed when investigating an inorganic biomaterial such as HA.

On the subject of bone tissue engineering and bone repair, in addition to the above-mentioned tests, another method, using simulated body fluids (SBF) is commonly practiced *in vitro*. This technique involves soaking bioceramics such as HA, β-tricalcium phosphate (β-TCP) and bioglass 45S5 in SBF to evaluate the ability of that particular bioceramic to form an apatite layer. SBF is a useful method to test the *in vitro* bioactivity of bioceramics and its reliability depends upon the type of bioceramics tested. For example, silicate ceramics have shown very good apatite-forming abilities in SBF while other bioceramics have shown no apparent sign of bioactivity when soaked in SBF for a short period of time. However, the bioactivity of the latter group has been demonstrated by *in viv*o studies. These findings suggest that although SBF is a good testing technique for some forms of bioceramics, it is however, not sufficient for other types such as phosphate-based or carbonate-based bioceramics and has to be used in conjunction with other methods. This phenomenon can be explained by the difference in the biochemistry of *in viv*o apatite and bone formation of these different types of bioceramics (*Evaluation of the In Vitro Bioactivity of Bioceramics*).

In the case of resorbable inorganic biomaterials such as resorbable bioceramics, *in vitro* biological assessment is an essential step to monitor the rate of resorption of the material. In most studies these materials are placed in a simulated aqueous humour which mimics the inorganic component of the human aqueous humour. In such studies, the degradation rates are calculated based on the release of calcium or phosphate from the bioceramics, such as HA or TCP, using different assays such as high-performance liquid chromatography (HPLC) and gel permeation liquid chromatography to measure the rate of degradation of the implant while inside the host. In addition to these, more accurate quantification can be obtained using HPLC designed to separate the various components of a solution, and to identify and quantify each of the separated

components. In the case of inorganic biomaterials they can be used to identify and quantify components of the implant (e.g., calcium) which have been released into the liquid system of their host after going through the degradation process.

In addition to investigating the biocompatibility, toxicity, organ formation (e.g., bone) and biodegradation, *in vitro* tests can be used to evaluate the material/device interaction with the cellular and protein components of blood. These tests, such as *thrombus assessment, coagulation assessment* and *platelets and platelet function assessment*, investigate the extent and site of thrombus formation, the presence of coagulation proteins (e.g., thrombin-antithrombin complex and fibropeptide A) and the number of platelets, respectively, when a material or device is placed inside the host. The interaction between the implant and blood has a significant effect on the biocompatibility of that implant. For instance, high levels of coagulation cascade activation observed during a coagulation assessment assay, may indicate unfavourable levels of device or material-related thrombin activity and fibre formation. (The most common tests used for *in vitro* assessment are summarised in **Table 3.2** and **Table 3.3**).

3.2.6.3 Summary

In vitro tests are the most frequently conducted because they are less complicated, cheaper and more reproducible compared with *in vivo* tests. In order to benefit from these advantages it is advisable to investigate different *in vitro* assays and choose the one that is most suitable for your particular application, and one that is in accordance with current rules and regulations set by the International Organization for Standardization (ISO) and the US Food & Drugs Administration. Once the assay has been chosen, decide on the cell type, number of cells, duration of exposure, test sample size, and the use of positive and negative controls to determine the viability of your test. Care should be taken when using information obtained from any of the mentioned assays as sometimes they can be misleading. This is especially true about the Ames assay, in which using fewer than four strains of *Salmonella typhimurium* can lead to false positive or false negative toxicity results.

3.3 *In Vivo* Assessment

3.3.1 Introduction

The *in vitro* environment does not mimic the physiological conditions present inside the body and thus to assess the true effect of materials, *in vivo* assessment of tissue compatibility or biocompatibility is necessary. This is performed to ensure material safety for in-human applications. Depending upon the stage of biocompatibility

testing, for example, early stages of material analysis or following promising results from *in vitro* experiments, it will be crucial to choose the right type of *in vivo* test, one that would provide the most relevant and precise information for the purpose of that particular investigation. *In vivo* testing is an important part of research as small or larger animal models are used to investigate a particular material and its effect inside its living host; therefore, avoiding unnecessary or unsafe direct human testing. *In vivo* testing ensures that materials are risk-free both in the short term (few weeks) and long term (few months or longer). *In vivo* animal studies are fundamental to understanding the mechanism of biomaterial interactions with living tissue as a whole, allowing for any unanticipated outcome to be found and rectified prior to conducting any in-human clinical studies.

3.3.2 Properties Assessed during In Vivo *Tests*

This section highlights tests which are considered for the general *in vivo* analysis of biomaterials based on the ISO standard recommendations. These include testing for cytotoxicity, sensitisation and irritation, based on ISO 10993-1. Cytotoxicity is, however, an *in vitro* test. A summary of these tests is listed in **Table 3.4** and schematically shown in **Figure 3.3**. The choice of *in vivo* test will depend upon the properties of the material and its end use as a biomaterial.

Table 3.4 Summary of various in vivo tests for biomaterial assessment, with a recommendation for animal models for each test

Medical device categorisation by nature of body				Biological effect									
Category	Animal model	Contact	Contact duration	Cytotoxicity	Sensitisation	Irritations or intracutaneous reactivity	Systemic toxicity	Subacute and subchronic toxicity	Genotoxicity	Implantation	Haemocompatibility	Chronic toxicity	Carcinogenicity
Surface device	Guinea pigs and albino rabbits	Skin	A	x	x	x							
			B	x	x	x							
			C	x	x	x							
		Mucosal membrane	A	x	x	x							
			B	x	x	x							
			C	x	x	x		x	x				
		Breached or compromised surface	A	x	x	x							
			B	x	x	x							
			C	x	x	x		x	x				
External communicating device	Mice/ rats/ rabbits	Blood path indirect	A	x	x	x	x				x		
			B	x	x	x	x				x		
			C	x	x		x	x	x		x	x	x
		Tissue/bone/ dentine	A	x	x	x							
			B	x	x	x	x	x	x	x			
			C	x	x	x	x	x	x	x		x	x
		Circulating blood	A	x	x	x	x				x		
			B	x	x	x	x	x	x	x	x		
			C	x	x	x	x	x	x	x	x	x	x

Implant device	Short term: Mice/rats/ rabbit Long-term: Sheep and pigs	Tissue/bone	A	x	x	x						
			B	x	x	x	x	x	x	x		
			C	x	x	x	x	x	x	x	x	x
		Blood	A	x	x	x	x	x		x	x	
			B	x	x	x	x	x	x	x	x	
			C	x	x	x	x	x	x	x	x	x	x

A: Limited (<24 h)

B: Prolonged (24 h – 30 days)

C: Long-term (>30 days)

Adapted from ISO 10993-1, *Biological Evaluation of Medical Devices - Part 1: Evaluation and Testing Within a Risk Management Process*, Geneva, Switzerland, 2009 [14]

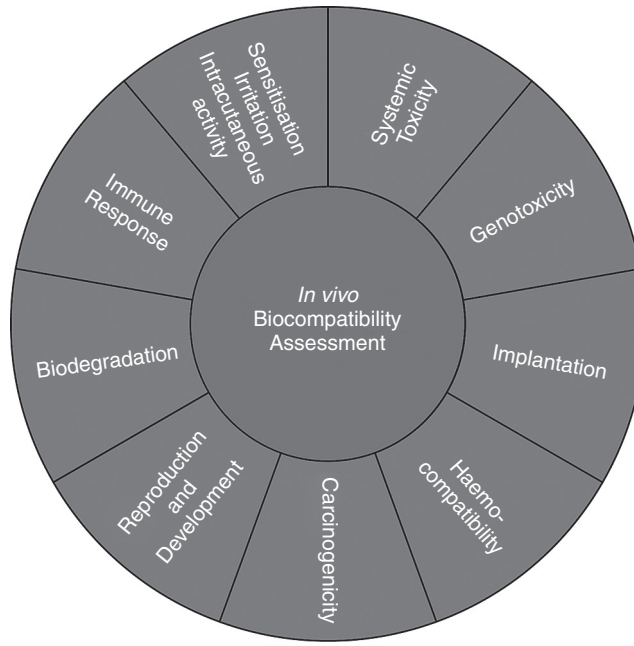

Figure 3.3 Components of *in vivo* biocompatibility assessment

3.3.2.1 Sensitisation, Irritation and Intracutaneous Reactivity

These tests mainly look at the effect of materials/devices or their leachability on skin tissue in terms of sensitisation or allergic reactions. Sensitisation or allergic reactions due to leachable chemicals are commonly tested on skin; whereas tests for irritation use extracts of biomaterials and look at their effect on local tissue. Intracutaneous tests, as their name suggests, are used to check the reactivity of intradermal tissue by injecting extracts of materials or devices.

3.3.2.2 Systemic Toxicity

Systematic toxicity tests look at the effect of new materials or devices, focusing on substances which may have leached into the body. Various chemical and physical properties of the material are considered when designing this type of test. Systematic toxicity tests can be categorised into four subsections; acute, subacute, subchronic and chronic toxicity, based on the duration it takes from introducing the test material into the animal model and for the adverse effects to be observed (**Figure 3.4**). Acute toxicity is considered to be those adverse effects observed after 24 h of administration of single or multiple doses of leachable materials, while subacute toxicity, and

subchronic toxicity refer to adverse effects presented after 14–28 days and up to 90 days, respectively. Furthermore, the extension of subchronic toxicity is termed as chronic toxicity which involves a period of over 90 days.

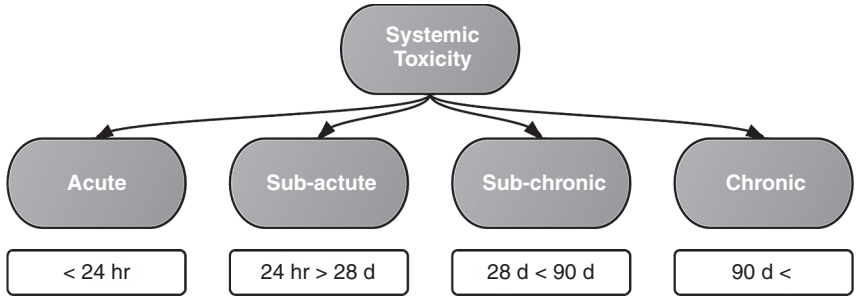

Figure 3.4 Division of systemic toxicity based on the duration of when the toxic response is observed in the host

3.3.2.3 Genotoxicity

Similar to the *in vitro* genotoxicity tests (**Table 3.3**), *in vivo* genotoxicity tests also look at changes in DNA which lead to the modification of cellular behaviour, such as proliferation and differentiation. *In vivo* micronucleus tests on rodents are the main type of testing techniques used to assess genotoxicity in living organisms. However, other tests such as the bone marrow cytogenetic test, chromosomal analysis, the rodent dominant lethal test and the mammalian germ cell cytogenetic assay can also be used. However, the choice of test will depend on the test material, its properties and procedural feasibility.

3.3.2.4 Implantation

This test is used to assess the possible pathological effect of the implanted material on the structure and function of the surrounding living tissues. The tissue surrounding the implant is analysed at a microscopic level using histological techniques. Commonly, immunological staining of histological sections is used to determine the type of cell surrounding the implant, collagen deposition and arrangement, and so on. Other information which is assessed during an implantation test include distribution and the number of inflammatory cells as well as their distance from the implant, and thickness and vascularity of the fibrous capsule. The quality and quantity of tissue growth inside or around the porous materials, tissue apoptosis, necrosis, cell proliferation rate, thrombus formation and endothelisation are other aspects that can be assessed during an *in vivo* implantation study. The choice of animal used for this purpose is

generally dependent on various factors such as the site of implantation, duration, size and the information required. Short-term studies of up to 12 weeks can be carried out in small animals such as rats, mice or rabbits, while long-term studies are normally conducted in large animals such as pigs, sheep or young cows.

3.3.2.5 Haemocompatibility

This test is used to study the effect of blood and its components on medical devices/materials. *In vivo* haemocompatibility tests are mainly designed to simulate the geometry, contact and flow dynamics of the tissue of interest. These tests are very important in the design of vascular biomaterials, heart valves and other similar devices. However, in general, haemocompatibility also provides information on the biocompatibility of medical devices/biomaterials which require contact with blood and are susceptible to an immune response, for instance, orthopaedic or dental biomaterials made of inorganic biomaterials, e.g., metal and essential minerals, such as calcium-based composites. The use of haemocompatibility tests are common and widely accepted for devices where thrombosis, coagulation and platelet activation have an important role to play in determining the success of the test material.

3.3.2.6 Carcinogenicity

Carcinogenicity tests are used to assess the tumourigenic effects of medical materials or their extracts. Tumourigenic effects caused by medical devices are not common, however, leachable materials can be considered as a likely cause of tumour formation. In addition, with the increased use of nano and microscale materials for biocompatibility coatings, their tumourigenic effect needs to be fully understood, using *in vitro* and *in vivo* assays, if the carcinogenicity of devices/materials are to be better evaluated.

3.3.2.7 Reproductive and Developmental Toxicity

These tests are designed to evaluate the effect of medical devices/materials or their extracts on the reproductive function, embryonic development, and prenatal and early postnatal development of living organisms. Tests and bioassays for this purpose are considered only when there is a possible negative impact on the reproductive potential of the host.

3.3.2.8 Biodegradation

This test is important for medical devices/materials which are likely to produce degradation products in response to the surrounding environment. Intentionally degradable biomaterials, such as degradable polymeric scaffolds, can leach out impurities, catalysts or other additives to the surrounding tissue and distant organs, while similarly, metallic and polymeric wear particles from the mechanical wear and tear of orthopaedic implants could also find their way into the surrounding tissues and organs, affecting their functionality. *In vivo* biodegradation tests can be designed to evaluate the effects of anticipated degradation products separately, and identify those that might impose potentially risky or negative impacts on the host tissues and organs. Specific tissue responses can be studied using histological analysis.

3.3.3 Immune Responses

Immune response evaluation is not part of the standard *in vivo* tissue compatibility assessment. However, efforts have been made to standardise protocols to document the effects of the immune response which are triggered by biomaterials and devices. Such tests are particularly important where modifications of natural biomaterial, for example, modified protein-based scaffolds, are suspected to induce immune toxicity. It should be noted that there is currently no specific guideline for testing the host immune response to inorganic biomaterials in place.

3.4 Case Studies

Raw material characterisation, *in vitro* and *in vivo* testing are all crucial components of a comprehensive study with the aim of developing a biocompatible product with medical applications in humans. Planning a workflow for the *in vitro* and *in viv*o assessment of biocompatibility is shown in **Figure 3.5**. The present two case studies on inorganic biomaterials can be used to demonstrate what information can be gained using any of these three types of studies and how this information can be interpreted in a comprehensive study with a medical application potential.

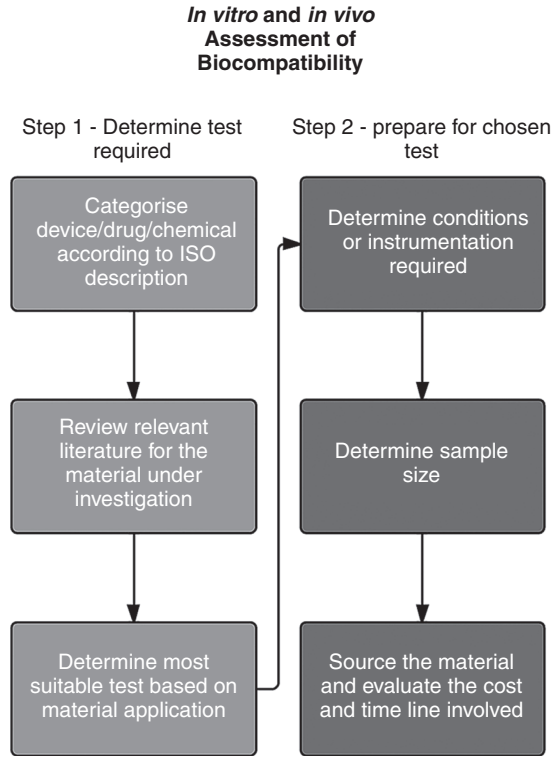

Figure 3.5 Planning a workflow for the *in vitro* and *in vivo* assessment of biocompatibility

3.4.1 Case Study 1

Inorganic biomaterials have many medical applications, one of which is treating cancer. Currently, radiotherapy is one of the most routinely practiced treatments for cancer. Although radiotherapy has been established as an effective method for treating cancer, it has become apparent that it is not as suitable for deep-seated cancers, as only a small dose of radiation penetrates deep enough to destroy this group of cancers. Furthermore, concerns exist regarding the potential damage to healthy tissues caused by radiotherapy. Ceramic microspheres might be the solution to these problems.

Previous studies have reported that 20–30 μm diameter $17Y_2O_3$-$19Al_2O_3$-$64SiO_2$ (mol%) glass microspheres are useful for the *in situ* irradiation of cancer [15]. This is achieved by neutron bombarding the yttrium-89 (89Y) in this type of glass to create the μ-emitter 90Y (half-life = 64.1 h). By implanting such activated glass microspheres

into the target tumour tissue, a larger localised dose of β-radiation is delivered to the cancerous area (**Figure 3.6**). In living tissue, β-radiation has a short penetration range (approximately 2.5 mm); therefore, the radiation emitted to the surrounding healthy tissue is minimal [16]. These glass microspheres, which have already been used in Canada, the USA and European countries to treat liver cancer [17], are particularly interesting because they exhibit high chemical durability. This high durability is important for their application in cancer treatment as it enables radioactive 90Y to remain within the microspheres inside the host and therefore, not affecting the surrounding healthy tissue whilst radiating the cancerous tissue.

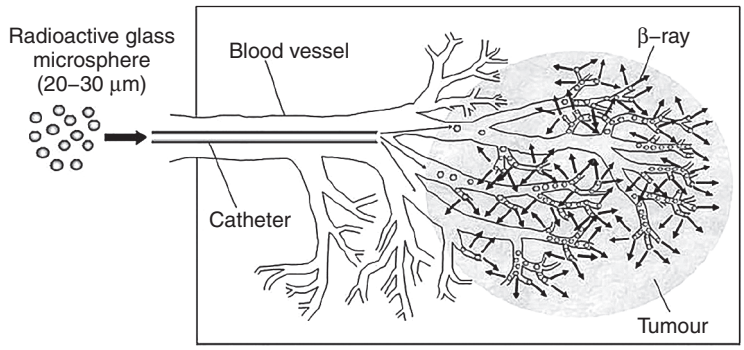

Figure 3.6 Schematic diagram of the local embolic radiotherapy of a tumour using yttrium-containing glass microspheres (20–30 nm in diameter). Reproduced with permission from Z.Z. Li and M.M. Kawashita, *Journal of Artificial Organs, 2011,* 14, 3, 163. ©2011, Springer [16]

In an attempt to treat cancer safely and effectively, resorbable calcium-phosphate ceramic microspheres have also been incorporated with TNP-470, an antiangiogenic agent, developing a novel chemoembolisation approach to target human solid tumours [17]. In this study, the human uterine sarcoma cell line FU-MMT-3 was used as a target since this type of tumour is very aggressive and unreceptive to contemporary radiotherapy or chemotherapy treatments. Here, FU-MMT-3 xenografts I nude mice were injected with calcium phosphate ceramic microspheres carrying TNP-470. The treatment was carried out three times per week for 8 weeks and the results showed suppression of tumour growth compared with the control treatments. A significant decrease in the intratumoural vascularity was also observed following the treatment with microspheres carrying TNP-470. This is probably due to the remarkable embolisation of the ceramic microspheres in the tumour microvessels and feeding arteries.

The use of ceramic microspheres for treating cancer has shown positive results for the effective and safe treatment of locally advanced or recurrent solid tumours, both *in vitro* and *in vivo*, during the above studies. However, the short half-life of these microspheres, their rapid decay even prior to cancer treatment, and their limited capacity to carry a large dosage of antitumour agents are important issues that need to be addressed by conducting further comprehensive studies. Furthermore, other independent *in vitro* assessments of ceramic microspheres, using common biocompatibility, toxicity and bioactivity assays, have indicated that these types of inorganic biomaterials are considered biocompatible, nontoxic and safe for various medical applications [18, 20, 21]. For instance, *in vitro* tests using DNA analysis results revealed that the microspheres might be cell biocompatible for a minimum of seven days when tested at a concentration of <7.5 g/l of medium [21]. The release profile of TNP-470 from ceramic microspheres was also investigated *in vitro*, which showed that almost 75% of TNP-470 was released within the first 30 min of immersion, while the remaining 25% was released at a slower rate taking up to 25 h [17]. Furthermore, inhibitory effects studied using *in vitro* assays, revealed that ceramic microspheres alone have no inhibitory effect on the proliferation of cancer cells, whereas those loaded with 500 or 1,000 μg/ml TNP-470 significantly inhibited the proliferation of FU-MMT-3 cells.

3.4.2 Case Study 2

Hydrothermal methods of synthesising HA have been around for a few decades now. Liu and co-workers [21] used a simplified hydrothermal method to synthesise powders consisting of crystallised HA and used various characterisation techniques including TEM, XRD, SEM and a microVickers indentation method, to investigate the powder morphology, crystalline phase, microstructure and mechanical strength, respectively. These techniques revealed the powder to consist of crystallised HA in a needle shape, 130–170 nm in length and 15–25 nm in width, while no decomposition was observed using XRD. SEM and mechanical testing revealed a pore-free surface structure with a flexural strength, microVickers hardness and a fracture toughness of 120 MPa, 5.1 GPa and 1.2 $MPa.m^{1/2}$, respectively.

A very similar principle of synthesis was adopted by a more recent study conducted by Okuda and co-workers [22], who compared hydrothermally synthesised HA (HHA) with rod-shaped particles against stoichiometric HA (SHA) with globular-shaped particles. They used various *in vitro* assays, to investigate the interactions of the bone cells on the surface of HHA and SHA, and *in vivo* histological studies, using rabbit models, to analyse the biological response of the host to HHA and

SHA. Using powder XRD with graphite-monochromatised CuKα radiation, they reported no phase other than HA for HHA and SHA, meaning that they were both pure and uniform. Furthermore, SEM and MIP were used to characterise the surface of HHA and SHA, as well as the pore volume and distribution of pore diameter, respectively. It was revealed that HHA was composed of micropores of 0.2 μm in size formed by tangled rod-shaped particles of about 20 μm in length, while SHA was composed of fused globular particles in the form of micropores of about 0.5 μm in size (**Figure 3.6**).

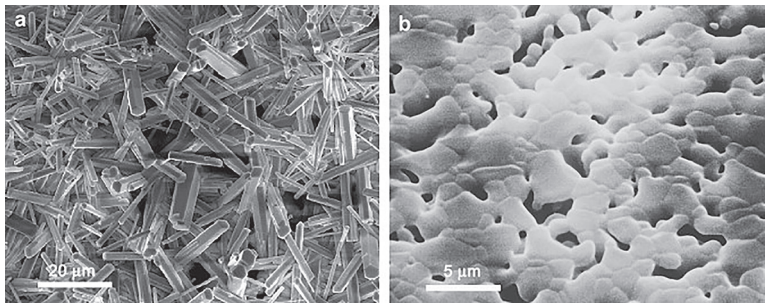

Figure 3.7 Scanning electron micrographs of the microstructure of (a) HHA, and (b) SHA. Reproduced with permission from T. Okuda, K. Ioku, I. Yonezawa, H. Minagi, Y. Gonda, Gi. Kawachi, M. Kamitakahara, Y. Shibata, H. Murayama, H. Kurosawa, T. Ikeda, *Biomaterials*, 2008, **29**, 2719. ©2008, Elsevier [22]

The *in vivo* studies, on the other hand, in which HHA and SHA were implanted into rabbit femurs, concluded that HHA had a significantly higher mineral apposition rate compared with SHA (**Figure 3.7**), suggesting that the microstructure of the inorganic biomaterials influenced the biological activity of osteoblast cells, even though they had exactly the same composition. Furthermore, the *in vivo* studies demonstrated that the bioactive response of the osteoblasts to HA nanoparticles is very much dependent on the particle size [22].

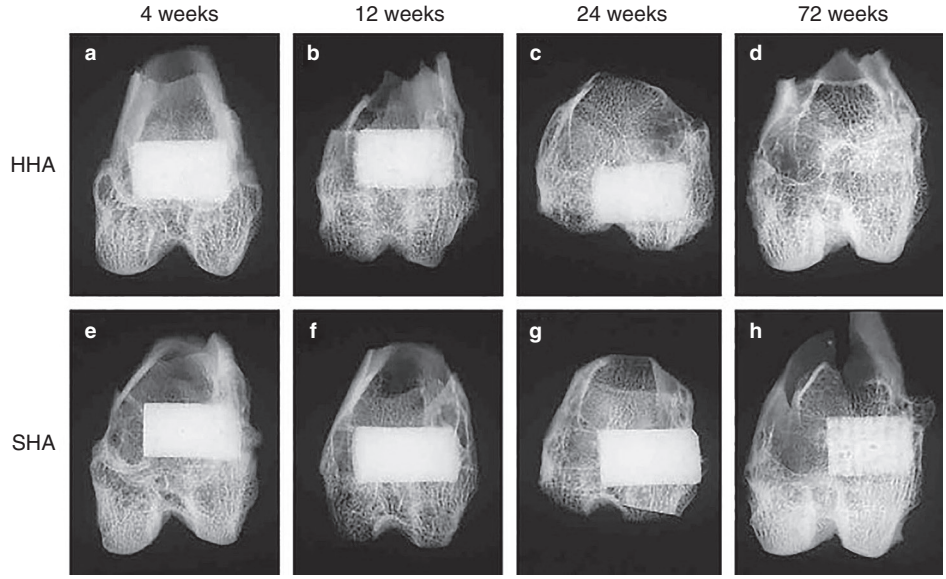

Figure 3.8 Soft X-ray photographs of the operated portion of the rabbit femur. (a) 4 weeks, (b) 12 weeks, (c) 24 weeks and (d) 72 weeks after implantation of HHA; (e) 4 weeks, (f) 12 weeks, (g) 24 weeks, and (h) 72 weeks after implantation of SHA. Involvement of HHA in bone tissue was evident at 72 weeks (h). Reproduced with permission from T. Okuda, K. Ioku, I. Yonezawa, H. Minagi, Y. Gonda, G. Kawachi, M. Kamitakahara, Y. Shibata, H. Murayama, H. Kurosawa, T. Ikeda, *Biomaterials*, 2008, **29**, 2719. ©2008, Elsevier [22]

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