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Characterization of scaffolds

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Abstract: Complete characterization of scaffold structure and properties is essentially the first step in the process of developing successful bone engineering scaffolds. Numerous studies demonstrate that pore size, interconnectivity and porosity affect bone tissue regeneration, and these three design features appear to be the most important structural variables in an initial scaffold screening. Clearly, besides scaffold morphology and mechanical and surface properties, biological characterization of scaffolds by suitable cell culture methods is also required.

Keywords: : *Scaffold, characterization.*

The most important function of a bone tissue engineering (TE) scaffold is its role as a template that allows cells to attach, proliferate, differentiate, and organize into normal, healthy bone as the scaffold degrades. Depending on the final application, scaffold requirements include matching the structural and mechanical properties with those of the recipient tissue and optimization of the micro-environment to support cell integration, adhesion and growth, issues that have become known as structural and surface compatibility of biomaterials. Scaffolds have to fulfil many requirements, such as osteo-conductivity, appropriate rate of biodegradation, interconnected porosity, suitable mechanical strength, and structural integrity. Therefore, the complete characterization of scaffold structure and properties is essentially the first step in the process of developing successful bone engineering scaffolds. Numerous studies demonstrate that pore size, interconnectivity and porosity affect bone tissue regeneration, and these three design features appear to be the most important structural variables in an initial scaffold screening. Clearly, besides scaffold morphology and mechanical and surface properties, biological characterization of scaffolds by suitable cell culture methods is also required.[1]

1. Structural (Architectural) characterization

1.a. porosity

Porosity is a measure of the void space in a material that can be determined from the ratio of the void volume to the bulk material volume. Porosity is known to play a role in determining cell seeding efficiency in addition to the diffusion properties and mechanical strength of a scaffold. However, it is not a unique parameter (i.e. structurally different materials can have identical porosities) and it cannot be used on its own to sufficiently characterize scaffolds.[1]

N.B: No single investigative technique is able to fully characterize the porous nature of scaffolds if they exhibit porosity in different scales, e.g. in some cases from nano to microporosity.

Characterization tools for scaffold porosities

Calculated either physical (manual) or automated image analysis

Porosimetry

Porosity assessment via porosimetry is based on the study of the flow of gases or liquids (or both), across a porous structure. Therefore, this method is only suitable for the

detection of open pores that allow fluid transport. Consequently, standard porosimetry methods cannot be used to assess total pore volume.[1]

i. Mercury intrusion porosimetry (MIP)

MIP Are based on the pressurized penetration of a liquid mercury into a porous structure and is a very useful method in characterization of macro-porous materials. Mercury has a high surface tension and therefore forms large contact angles with most other materials (~=130◦). In addition, mercury does not spontaneously penetrate pores by capillary action, and therefore an external pressure must be applied to force mercury into the pores. The pore diameters (size) intruded by mercury can be calculated according to the following equation:

$$D = \frac{-4\gamma\cos\theta}{p}$$

where D is the diameter of the pore, γ is the surface tension of mercury, θ is the contact angle between mercury and the solid and p is the applied pressure.

The direct data acquired is the accumulated volume of mercury entering the porous system. A pore size distribution curve is obtained by relating the log differential intrusion volume to log pore diameter. [2]

Limitations:

1. As with flow porosimetry, closed pores are hidden from the test.
2. Fibrous meshes are susceptible to undergo mechanical deformation under the pressures attained in a mercury porosimetry experiment.
3. Assuming that pores are cylindrical is often an over-simplification. It may not provide an accurate surface area measurement, due to a well-known problem associated with the so-called "bottle neck" effect. This occurs where large cavities exist behind narrow necks, which are considered to be pores having the diameter of the neck.[2]
4. Mercury health hazards: exposure to mercury may cause irritation to the eyes, skin, and stomach; cough, chest pain, or difficulty breathing, insomnia, irritability, indecision, headache, weakness or exhaustion, and weight loss. Workers may be harmed from exposure to mercury.[3]

N.B: Liquid intrusion porosimetry is equivalent to MIP, with the exception that other non-wetting liquids such as oil or water are used. Due to the lower viscosity of the liquids applied here and the pressures needed, smaller pores may be measured, as small as 1 nm.[4]

ii. Liquid displacement method:

Liquid displacement is a method often used to characterize scaffold porosity. In the liquid displacement method, the scaffold is added to a known volume of liquid V1, commonly ethanol, and is often assisted by various techniques to ensure that all pores are completely filled with liquid.

The resulting volume V2 is measured, and the impregnated scaffold is removed from the container. The volume of the liquid in the bath after scaffold removal V3 is recorded, and this volume is equivalent to the void volume. From these measurements, porosity can be calculated according to Equation:

$$\text{porosity} = \frac{V3}{V3 + V2 - V1}$$

N.B: This method was also used with hexane as a displacement liquid due to ethanol potentially shrinking the silk scaffold.[4]

iii. Liquid pycnometry (Archimedes principle):

Liquid pycnometry follows the same steps as the liquid displacement method, with the exception that instead of measuring volumes, weights are measured. The whole procedure takes place in a pycnometer filled with a liquid. First, the weight of the liquid W_1 and the dry weight of the scaffold W_s are measured, then the scaffold is inserted into the liquid. Once this has been achieved, more liquid is added to compensate for the liquid initially displaced by the scaffold until the pycnometer is full again.

The weight of liquid and scaffold together W_2 is recorded, and then that of leftover liquid when the scaffold has been extracted W_3. The porosity can be calculated using Equation: [4]

$$\text{porosity} = \frac{W2 - W3 - Ws}{W1 - W3}$$

iv. Gas pycnometry:

Used to measure the volume of solids based on Boyle's law (PV=K), where P is pressure, V is volume & K is constant. The most basic setup for this approach requires a reference chamber of a known volume connected by a valve to a sample chamber, and a manometer (pressure measuring tool) associated to each chamber. First, the pressure of both chambers is measured. Then, the valve connecting the chambers is opened, allowing the pressure to reach an equilibrium.

Where p1C is the initial pressure of the chamber containing the sample, V1 is the gas volume in the chamber, V2 is the volume of the reference chamber, p2C the initial pressure of the reference chamber, and pO is the equilibrium pressure reached after the valve is opened.

The volume of the sample can be calculated. Gas pycnometry allows quantitative assessment of scaffold porosity. The approach to convert the volume to porosity is by measuring the apparent volume of a cube that the scaffold has been cut into, using a caliper. The measured pycnometer volume is then inverted and divided by the apparent volume, the result being porosity, as shown in Equation: [4]

$$\text{porosity} = \frac{V_{\text{Apparent}} - V_{\text{Pycnometer}}}{V_{\text{Apparent}}}$$

Closed pores cannot be characterized by gas pycnometry.[4]

v. Gas adsorption:

Based on The Brunauer–Emmett–Teller (BET) Theory in which a gas, commonly N2, adsorbs to the surface of a measured solid and provides a quantitative assessment of its specific surface area. The data acquired by BET measurements have the form of isotherm curves providing information on each adsorption layer. These adsorption layers are schematically shown in (Figure 2i), The resolution of this method is extremely high, since it can characterize the surface area of pores as small as 0.5 nm (but only as large as 2 μm).

N.B: The curve showing the relation between pressure and volume of a given mass of gas when the temperature is constant is called its isothermal curve. It is represented by the equation. PV= constant. [4]

Methods to visualize and subsequently quantify porosity in scaffold structures are:

i. Scanning electron microscopy (SEM):

Scanning electron microscopy is a method in which the surface of a sample is examined, providing information on its morphology. Commonly, to achieve this, an electron beam is directed at the sample, which then excites the atoms on the surface, causing secondary electrons to be emitted. These are then detected, and an image can be constructed. Samples are often nano sputtered with gold, platinum, or other conductive materials to render the surface conductive and to avoid charging effects. Further preparation steps are often taken in order to ensure the sample is dry.

An advantage of SEM over physical methods is that it can qualitatively assess cell growth on surface layers. SEM has also been used to characterize scaffold porosity by converting images to binary (back scattered), whereby fibers are black and pores are white or vice versa.

Limitation: These images are a 2D projection of a 3D pore structure. Its limitation is what might appear to be a closed pore in 2D could actually be an open pore in 3D.

One extremely different approach consisted of using a focused ion beam (FIB) to remove surface layer coating and progressively take 2D SEM images of the subjacent layers,

allowing for subsequent 3D tomography. It was concluded that the 2D imaging was insufficient in providing insight into the true morphology of the scaffold, however, the 3D method caused results with much noise and was not compared with other 3D characterization methods.[4]

ii. TEM:

Transmission Electron Microscopy (TEM) is a microscopy technique that is equivalent to light microscopy, except it uses electrons instead of photons, i.e., light, thus allowing for a much higher resolution due to the easily achievable small wavelengths.

Limitation: However, the optimal thickness for studied samples is in the low nm range, rendering it unsuitable for many scaffolds, and only having the potential to examine individual fibers containing nanomaterials.

iii. AFM:

Is a technique that is used to map the topography and to study the properties of materials on micro and nanoscales. AFM uses a probing tip at one end of a cantilever to interact with the material (sample). The interaction between the sample and the tip gives rise to either attractive or repulsive forces.

It can operate in contact, non-contact or tapping mode. The forces exerted on the cantilever by the surface of the sample can then be characterized by a laser-detector setups. These forces give information about the topography of the sample with resolutions of under 1 nm. If the tip and the sample are close to each other, the attractive force deflects the cantilever towards the sample, and when the tip is brought into contact with the sample, the repulsive force detects the cantilever away from the sample.

A laser beam detects these deflections, any deflection will cause changes of the direction of the reflected beam. The most popular ways of obtaining topography imaging are contact and tapping modes. The tapping mode, in comparison with the contact mode, presents the advantage of reducing friction forces when scanning (soft) samples.

One advantage of AFM is that it allows for the characterization of not only morphological, but of mechanical properties.[4]

iv. Confocal laser scanning microscopy (CLSM):

CLSM is a technique that allows for filtering and increased resolution of light microscopy. It can be combined with fluorescence microscopy to increase the resolution and is therefore another powerful tool to image cell growth. Due to the ability of CLSM of obtaining 2D images at different depths, a 3D image can be reconstructed.

Limitation: Confocal microscopy requires fluorescence staining of collagen, which may affect cell–matrix interactions because staining can change the density of ligands on the surface of scaffold.[5]

v. Computed tomography (μ -CT):

Micro Computerized tomography (CT) is a technique in which X-rays are used to create a 3D image of the structure of a sample. It is a **qualitative** and **quantitative**, **non-destructive** method for obtaining the fiber diameter and alignment, porosity shape, pore size and interconnectivity and distribution all from one measurement by providing a complete slice by slice 3D scaffold representation. Pixel has a side size in the μm range. This allows for the characterization of μm-scale structures. μ-CT can access both connected and isolated pores enabling the **total void volume** to be determined.

Can scan all types of materials in hydrated or dried state (i.e., polymers, ceramics, metals, and composites) obtained through various fabrication methods (e.g., membranes, fibers, porous scaffolds, particles). However, the visualization of certain materials is somewhat problematic. Among them, polymers that exhibit low X-ray absorbency. [6]

To improve their contrast in CT imaging—in either dry or hydrated state—several staining agents typically used in histology were tested as barium chloride, iodine, potassium iodide, and silver nitrate as contrast. 1.5 wt.% barium chloride is the ideal amount in order to preserve the initial morphology and improve the image contrast.[6]

Nano-CT uses the same technology as micro-CT, with the pixel sizes being in the nm range, making it a tool capable of characterizing many features of nanoscale scaffolds, however, many research institutions lack access to nano-CT equipment.[4]

N.B: Other imaging techniques, such as scanning electron microscopy (SEM) associated with EDX, atomic force microscopy (AFM), or confocal microscopy (CFM), provide important information regarding surface morphology, topography, and chemical composition. Nonetheless, their use is associated with several drawbacks, such as the need to destroy the sample to obtain suitable geometries that can be further analyzed, and the registered data provide information only with respect to the surface of the sample (SEM, AFM) or thin 3D sections (CFM).[6]

1.b. Permeability

Successful bone TE depends on the scaffold's ability to allow nutrient diffusion to and waste removal from the regeneration site, therefore, permeability is a key parameter for the design of scaffolds. Permeability is directly related to the degree of pore interconnectivity. Several permeability measurement systems have been developed for determining the permeability of scaffolds.

Characterization tools for scaffold permeability

i. Intrinsic permeability testing

The physical principle used for the measurement of permeability is based on the measurement of the pressure drop caused by the introduction of the scaffolds in a fluid (e.g. water or cell culture medium). Immediately prior to intrinsic permeability testing, each scaffold's length and diameter is measured using digital calipers. Scaffolds are then carefully wrapped with Teflon® tape and placed into a custom-built flow-rate-controlled permeability chamber. The chamber is connected with a flow-through blood pressure transducer and syringe pump.

Deionized water was then pumped through the sample at a constant flow rate, and the pressure drop across the sample was recorded until the system reached steady state. Intrinsic permeability can be easily determined by applying simple mathematical relations. According to Darcy's law which states that states that the flow rate through a sample is proportional to the applied pressure, intrinsic permeability k [m2] can be obtained by: [7]

$$K = \frac{Q \mu L}{A (Pb - Pa)}$$

where Q is the flow rate (m^3/s), A is the cross-sectional area (m^2), Pb - Pa is the drop pressure between two points, μ is the dynamic fluid viscosity (Pa s), and L is the scaffold thickness.

ii. MRI methodology

High-resolution MRI is used for characterizing the permeability and fluid velocity. The sample is sealed in a specially constructed MRI probe consisting of a flow chamber surrounded by a solenoid RF coil (electromagnetic coil produces uniform magnetic field) and positioned in the MRI scanner.

Cross-sectional fluid velocity images are generated using a phase-encoding MR imaging technique. A whole-body MR scanner (equipped with a spectrometer is used to obtain MR image as a 3-D complex matrix.[8]

1.c. Crystallinity

Processing parameters are known to affect the polymer crystallinity. When processing PCL using solution electrospinning the extremely rapid removal of solvent may be expected to result in little opportunity for crystal nucleation and hence poor crystal

structure. In contrast, drawing of the fibers in the whipping region of the jet enhances the crystallinity and orientation of the polymer chains.

The crystallinity of scaffolds can be characterized by:

i. X-ray powder diffraction (XRD)

Is a rapid analytical technique primarily used for quantitative determination of crystal phase content and composition and can provide information on unit cell dimensions. Based on the ability of crystals to diffract X-rays in a characteristic manner allowing a precise study of the structure of crystalline phases. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings.

It is used to study the crystalline content, identify the crystalline phases, spacing between lattice planes, and epitaxial growth of crystallites. Since every material has its unique diffraction patterns so materials and compounds can be identified by using a database of diffraction patterns. The wide angel XRD (WAXD) (diffraction angle greater than 5) technique is used to determine the degree of crystallinity of polymers and fibers. It can also be used to determine the chemical composition or phase composition of a film, the texture of a film, the crystallite size and presence of film stress.[9]

2. Mechanical characterization

Clearly, understanding the correlation between pore structure, porosity, and scaffold mechanical properties is crucial in the process of optimization of scaffold architecture. The regeneration process is also influenced by the mechanical properties of the scaffold. An artificial substrate conveys to cells' physical signals (e.g., stiffness) that regulate many processes in regeneration, such as cell proliferation and migration. For this reason, the mechanical compatibility of the material is fundamental in determining the outcome of the regeneration process and the scaffold would rather resemble the mechanical properties of the native tissue.[10]

Accurate measurement of mechanical properties of scaffolds for biomedical applications is essential, to guarantee they can withstand the forces during surgical operation and those exerted by physiological activities and/or by tissue growth. [10]

The mechanical properties of scaffolds can be characterized by:

- The standard mechanical tests are **uniaxial tension, compression, and indentation**. In most of the cases, compressive mechanical testing is used to measure the mechanical strength of a scaffold. For a tensile test to be accurate and repeatable it is important to report macroscopic dimensions (gauge length and cross sectional area), the strain rate, the applied load, as well as whether they have been performed at room temperature or under physiological conditions (at 37 C, in PBS or culture media).
- Uniaxial tensile testing gives information about the Young's Modulus or stiffness (E) in tension (slope of the initial linear stress-strain), yield strength (end of linear elastic region, beginning of non-linear plastic region), fracture stress/deformation, and fracture energy (toughness) per volume.
- Other techniques such as AFM-based nanoindentation or bending tests have also been reported to measure the local stiffness, hardness and flexural properties. [10]

i. Compressive strength testing:

Studies usually report the measurement of compression strength via uniaxial testing. Specimens are compressed between two fixed steel plates at a rate of typically 1.0 mm/min. In case of swollen samples, the platens may be permeable to allow the escape of water as the sample is compressed. The load versus displacement curve was obtained through a computerized data-acquisition system.

The compressive strength of the structure may be calculated as as the maximum applied load (carried at the 0.2% offset) divided by the cross-sectional area of the sample. Thus, the compression strength test characterizes the mechanical properties of porous ceramics, hydroxyapatite, bioactive glass and composite scaffolds. The area under the

stress–displacement curve obtained in a compression strength test is usually considered to access the work of fracture, a measure of the toughness of the scaffold. [10, 11]

ii. Tensile strength testing

Mechanically characterized by uniaxial tensile tests at a constant cross-head speed using an Instron (universal) Testing System. The simplest measurement that yields the elastic modulus of a specimen, in which the sample is grasped at two ends and pulled while axial strain and stress are simultaneously measured. Samples are tested at a specific strain until **specimen failure**. By analyzing the obtained stress-strain curves, the Young's modulus (MPa) can be calculated.

N.B: If the sample is anisotropic, additional uniaxial tests in the other two coordinate directions can be used. Stresses can be applied in two (biaxial) or three (triaxial) dimensions simultaneously.[10, 11]

iii. Fatigue

Fatigue can be tested using an Instron testing machine under uniaxial cyclical loading.

iv. Nano-Hardness

Nano-indentation is the most common characterization technique used, chiefly because it allows the hardness of specific areas of an electro-spun membrane to be evaluated with very fine spatial resolution and with minimal preparation.

However, there are problems with precision and sometimes accuracy when the technique is applied to polymer surfaces, especially fibrous and porous ones. Many polymers are too soft to be investigated using nanoindentation, while viscoelastic behaviour (creep) and difficulty in accurately characterizing tip shape prevents accuracy using the traditional analysis of Oliver and Pharr.

Compared with other methods, modulus values of polymers measured by nanoindentation are often much larger or even negative in extreme cases, because of the effects of creep. Recent attempts to characterize the creep response of polymeric materials suggest that the modulus depends on the speed of the indentation and thus there is no single well-defined value for modulus in these materials. The most suitable technique used for the nano-indentation of polymers, which can be related to electrospun membranes include AFM.[2]

N.B: specimens for nano-hardness measurement must be non-porous.

Dynamic indentation

Attempts to characterize the viscoelastic behaviour of polymer samples have also been made using dynamic indentation with an oscillating tip. Storage (elastic part) and loss (viscous part) moduli were determined in studies of different polymer materials.

A hemispherical indenter impacts a disk at velocities from 100 mm/s to 5000 mm/s, typically deforming the specimen to failure. Displacement of the impact head is measured using a high speed video camera and Digital Image Correlation (DIC). We are not aware of studies involving dynamic indentation on porous surfaces but this method does have potential for characterizing storage and loss moduli of electrospun membranes. [2]

Problems faced when applying nano-indentation to fibrous membranes

- There are obvious difficulties for determining the moduli of porous samples using nano-indentation. These include ensuring both an ideal contact between tip and fibre and that the fibre is adequately supported to prevent it bending or slipping away from the probe.
- A more common method is to test a single fibre, which has been spun onto a hard plate. In this situation it was found that the substrate on which the fibre is mounted can affect the measurement if the fibre diameter is below 300 nm.
- Problems involving tip contact with a small-radius fibre; the probe may not contact at 90◦ or it may slip slightly.[2]

Atomic force microscope based on indentation

Imaging is not the only feature from atomic force microscopes. AFM devices can also be used as a "mechanical" machine. In these experiments, an AFM-tip or a colloidal probe

is extended and retracted towards/from the sample of interest. Such motion takes place under controlled displacement speeds. During this process, the deflection of the cantilever is determined as a function of the displacement of the scanner, and the force sensed by the cantilever is calculated using **Hooke's law**. The force-distance curves recorded in this way can be divided into three clearly distinguishable segments (approach, contact with the sample, and retraction). They can be described as follows:

1. The approach curve delivers information about the existing repulsive or attractive forces between the tip/colloidal probe and the sample (e.g., electrostatic, van derWaal forces). These types of measurements have been crucial for the understanding of molecular and colloidal interactions.

2. The second part of the curve, during contact between the cantilever and the sample, provides information about (e.g., Young´s Modulus, stiffness, relaxation time, hardness and viscosity).

3. Finally, the segment representing the retraction motion relates to adhesive forces. The maximum adhesion parameter, or pull-off force, is indicative of the stickiness of the sample.[2]

3. Characterization of surface wettability [12]

Hydrophobic materials are typically characterized by large contact angles (CAs) often (>90°), whereas hydrophilicity is characterized by low CAs (<90°). Routine water CA measurements using the **sessile drop technique** feature the deposition of droplets of water using a syringe and needle controlled by a syringe pump to deposit the water at a nominal flow rate or to produce a standardized volume. Once the water droplet falls onto the experimental material below, a back-lit image of the droplet on the surface in profile is captured by a **high-resolution camera/video contact analyzer**.[24] The contact angle is measured 5 times from different positions on each scaffold and an average value is calculated.[23]

A change in the **contact angle** can be a useful indicator of successful surface modification or blending of the scaffold to improve the wettability, however, the contact angle is also dependent on the surface roughness and porosity. When a droplet of water is placed on a fibrous mesh only a fraction of the water comes into contact with the fibers which decreases the liquid–solid interactions and increases the liquid–air interactions leading to typically higher contact angles than for smooth surfaces. In addition, when measuring contact angles of meshes it is often difficult to extrapolate the circular part of the drop profile with the surface when it is irregular.[12]

For these reasons the water contact angle of PCL meshes/scaffolds can vary greatly and the tested specimen should be non-porous, flat smooth solid surface which presents a challenge in biomaterials research. When PCL is modified with collagen, gelatin or plasma the contact angle reduced to zero meaning it often only serves as a qualitative guide to the success of a modification process when used in this way.[12]

On **porous substrates** an equilibrium between drop and surface is not reached, thus a **dynamic contact angle** is measured. It was experimentally proven that the **advancing** and **receding** contact angles, and **the contact angle hysteresis** of rough and chemically heterogeneous surfaces, are determined by interactions of the liquid (using syringe-needle method). The "advancing contact angle", θa, and "receding contact angle", θr, values are also measured to express the effect of the actual surface roughness and chemical heterogeneity of the substrates. θa indicates the contact angle when the volume of the droplet is expanded through a syringe (or a dispenser) the three-phase contact line is advanced on a fresh substrate. Meanwhile, θr designates the contact angle when the volume of a preformed droplet on a substrate is withdrawn by applying the suction of a portion of liquid from the droplet through a needle, showing the minimum contact angle value before the three-phase line is broken inwards. **Contact angle hysteresis (CAH)** is the difference between θa and θr. CAH = 0 and θ = θa = θr on ideal, atomically flat and chemically homogeneous surfaces. In practice, CAH is around 5–20° on most of the practical surfaces. CAH

value depends on the magnitude of surface roughness and the surface chemical heterogeneity of solids.[27]

Measurement setups employ a camera recording images of the drop and measuring the contact angle from the images. Once the liquid is released onto the substrate surface the instrument starts image acquisition of the drop at different rates. Drops falling on the substrate initially oscillate, stable measurement of the contact angle is only possible after the kinetic energy has dissipated. The image analysis of contact angle instruments also measures the base diameter of the drop and the drop volume over time. In this way not only the contact angle is evaluated for each frame taken, but also the remaining quantity of liquid on the substrate. All contact angle measurements are carried out under standard climate conditions, 23 °C and 50% relative humidity.[25]

Similarly to contact angle analysis**, XPS** has been used only qualitatively to identify new chemical species for surface modified PCL including increased oxygen presence after air plasma, increased nitrogen after inclusion of collagen, –OH and –CO after argon or oxygen plasma.[12]

4. Biological characterization

Scaffolds for bone tissue engineering need to be characterized **in-vitro** by cell culture methods before **in-vivo** and **clinical studies** take place. The cost of in vivo animal studies and the loss of animal lives continue to motivate the development of in vitro screening assays.

In vitro biological characterization of scaffolds:

The in vitro investigations can be divided into two levels:

a. **The first level involves analyzing cyto and structural compatibility of the scaffold materials with selected cell lines**

The first question regarding application of scaffolds for bone TE is the cyto compatibility of the newly developed material. In screening tests, the effect of porous scaffolds on the functions of cell types is investigated by **continuous cell lines**. Different cell types are available for measuring cell behavior on biomaterials for bone regeneration, for example, mouse calvaria osteoblast-like cells and human osteosarcoma cell lines. During various incubation periods of 24 h up to 3 days. Essential parameters are determined, like:

1. Cell **morphology**, are determined using **light or scanning electron microscope imaging.**
2. **The number of attached cells** (by intracellular LDH activity or BrdU assay).
3. **The cell viability** (by mitochondrial activity, MTT or WST-1) are analyzed.

Dynamic bioreactors concepts as **spinner flasks** and **rotating wall vessel** have been developed to mimic the native micro-environment during cell culturing. **Spinner flasks** support the 3D cell culture by continuously stirring cell suspensions. 3D scaffolds can be integrated within the spinner flask systems that permit the seeding and penetration of cells within the scaffolds.

Due to the continuous stirring, spinner flasks generate local high shear regions that damage cells or the integrity of MCS. Therefore, **rotary wall vessel bioreactors** were developed to reduce shear stress by rotating the cell culture chamber rather than stirring the cell culture media. Both bioreactor systems enable enhanced mass transport of nutrients.[1]

b. **The second level considers the interactions of cells and scaffolds, cell attachment, cell proliferation and osteogenic cell differentiation**

There are a number of different types of in-vitro osteogenesis assays currently used that attempt to predict in-vivo performance listed here in historical order

1. In vitro apatite forming ability measured by a simulated body fluid test.

(Its idea is that materials forming an apatite layer on their surfaces are able, in principle, to bond to bone. They also speculated that this apatite formation is reproducible in-vitro

and invented an acellular simulated body fluid (SBF) in which the ion concentrations and pH were nearly equal to these factors in human blood plasma. They found that the in-vivo apatite formation was successfully reproduced in-vitro when these materials were simply soaked in SBF at 36.5 °C. They proposed that the bone-bonding capability of a given material could be evaluated by examining the apatite-forming capability on its surface in SBF).[13]

2. Invitro osteogenic differentiation assays involving seeding of human or rodent osteoprogenitor cells such as MSCs, calvarial bone progenitors, or cells lines derived from an osteosarcoma and evaluating their differentiation via bone protein expression (bone sialoprotein, osteocalcin), alkaline phosphatase and mineral content which represent typical markers to identify osteoblasts.[13]

3. Imaging to determine the cell behavior on the scaffold including cellular attachment, spreading & differentiation.

- **SEM** it can qualitatively assess cell growth on surface layers.
- **μ-CT & Nano-CT** has excellent penetration depth. However, X-ray radiation is ionizing and can damage tissue or samples.
- **MRI** has excellent imaging penetration depth and safety. MRI can provide anatomical, functional, and cellular information.
- **Immunofluorescence microscopy & Confocal laser scanning microscope** has high sensitivity and excellent spatial resolution. Also, various types of biomarkers can be easily used with optical imaging to monitor intracellular signaling and cellular interactions.
- **Multimodal imaging** can be one strategy to overcome limitations of each imaging method and complementarily offer morphological, functional, and molecular information about tissue-engineered constructs. In addition, the multimodal imaging strategy tends to utilize synergetic features of different imaging techniques. Recently, combinations of imaging modalities such as MRI/CT and MRI/fluorescence have been explored for visualization of engineered tissue constructs in preclinical and clinical applications. In addition, contrast agents are also essential for multimodal imaging and various types of multimodal imaging contrast agents have recently been developed.[26]

5. Chemical characterization

Chemical characterization of scaffolds and chemical composition can be obtained by:

i. Fourier Transform Infrared Spectroscopy (FTIR) [14, 15]

FTIR is a useful and convenient tool for determining the chemical composition of scaffolds. It is used to study and identify chemical substances or functional groups in solid, liquid, or gaseous forms. When used with a total internal reflectance (ATR) accessory it provides a quick, semi-quantitative method for confirming the presence of additives as nano-hydroxyapatite (nHA) or gelatin. The ATR method is normally considered non-destructive, however, good contact between the sample and ATR crystal requires applying significant pressure which will damage delicate scaffold morphologies.

FTIR is also useful for determining the protein conformation (which is protein held together by different bonds and folded into a variety of three-dimensional structures. The folded shape, or conformation, depends directly on the linear amino acid sequence of the protein) based on characteristic shifts of amide groups indicative of hydrogen bonding.

Samples should therefore be ground in a mortar to reduce the average particle size to 1 to 2 microns. About 5 to 10 mg of finely ground sample are then placed onto the face of a KBr plate, a small drop of mineral oil is added and the second window is placed on top. With a gentle circular and back-and-forth rubbing motion of the two windows, evenly distribute the mixture between the plates. The mixture should appear slightly translucent, with no bubbles, when properly prepared.

IR Advantages:

1. All kinds of material can be analyzed.

2. IR can provide a molecular fingerprint that can be used when comparing samples. If two pure samples display the same IR spectrum it can be argued that they are the same compound.

3. IR is most useful in providing information about the presence or absence of specific functional groups.

4. Fast, easy and less expensive.

5. Very small amount of sample is required.

ii. X-ray Photoelectron Spectroscopy (XPS) [16, 17]

The most commonly used surface chemical analysis technique for polymers and biomedical materials is XPS. It can identify the elements that exist within a material (elemental composition) or are covering its surface.

XPS is a powerful measurement technique because it not only shows what elements are present, but also what other elements they are bonded to. Each element produces a set of characteristic XPS peaks. These peaks correspond to the electron configuration of the electrons within the atoms, e.g., 1s, 2s, 2p, 3s, etc. The number of detected electrons in each peak is directly related to the amount of element within the XPS sampling volume.

Advantages of XPS:

1. Non-destructive.
2. Surface and elemental sensitivity.
3. Characterize all elements (except H & He).

Disadvantages of XPS:

1. Expensive.
2. Samples must be compatible with high vacuum environment. So, if your sample will outgas when placed under vacuum, XPS is not the right test for your needs.

iii. SEM & Energy Dispersive X-ray Spectroscopy (EDX) [18]

Scanning electron microscopy (SEM) is an effective method in analysis of organic and inorganic materials on a nanometer to micrometer (μm) scale. In scanning electron microscopy (SEM) the surface of a specimen rather than its interior is scanned with an electron beam.

Energy Dispersive X-ray Spectroscopy (EDS) works together with SEM to provide qualitative and quantitative results. The device consists of variable pressure system with the ability to hold any samples (even wet or samples with minimum preparation). The EDS added the advantages of evaluating the composition of various elements in the sample by converting the intensity of x-ray ratio to chemical compositions in a few seconds.

6. Characterization of Scaffold Degradation

i. Characterization of the Biodegradation Process In-vitro [19]

In vitro **hydrolytic degradation** study is typically carried out in PBS at a pH of approximately 7.2 or simulated body fluid (SBF) at 37 °C so as to mimic the aqueous in vivo environment. **Oxidative degradation** of polymers is studied by immersing the polymer in PBS containing CaSO4 and H_2O_2. For an in vitro **enzymatic degradation** study, the sample is typically incubated with lysozyme or MMPs in a simulated physiological environment of pH7.4 and 37 °C.

Due to the long degradation time of certain synthetic polymers, an accelerated in vitro degradation study is sometimes carried out to predict long-term properties of a scaffold by elevating the temperature and/or adjusting the pH conditions. However, note that the degradation mechanisms in acid, alkaline and neutral environments are different, and the elevated temperature can often affect the crystallinity of the polymer. For these reasons, the relevance, applicability, and validity of an accelerated degradation study need to be carefully reviewed.

Optical and electron microscopy techniques are typically used to study the size, shape, and surface morphology of tissue engineering scaffolds. Light microscopy is

sometimes sufficient to measure the changes in size and shape of the scaffold structure. When the scaffold is significantly thick, light sectioning techniques such as laser scanning confocal microscopy, light sheet or multiphoton methods are needed to obtain a clear image of the features of interest.

During degradation, the morphology of the scaffold changes significantly due to liquid imbibition, surface erosion and molecule rearrangement. When such changes are hard to detect with light microscope, then scanning electron microscopy, transmission electron microscopy, and atomic force microscopy are helpful. Mass or weight loss is one of the three main polymeric factors that is directly affected by degradation.

It is commonly characterized by measuring the dry weight of the sample before and after a certain degradation period using Equation:

$$\text{Mass loss\%} = \frac{W0 - Wt}{W0} \times 100\%$$

where Wt is the dry weight of the sample after a certain degradation period and W0 is the initial dry weight of the sample.

N.B: The test is destructive due to the process of sample dryness, therefore, the sample cannot be used to measure degradation at different time intervals.

Fourier transform infrared (FTIR) spectroscopy can be used to detect the cleavage or scission of functional group along the polymer chain during degradation, and Ultraviolet visible spectroscopy (UV-Vis) can be used to detect the decomposition of the polymer's carbon structure.

Degradation occurring initially in the non-crystalline regions, the level of crystallinity in synthetic polymeric scaffold is expected to increase (increased crystalline/amorphous ratio) during early periods of degradation. These changes can be characterized by X-ray powder diffraction patterns (XRD).

ii. Characterization of the Biodegradation Process In-vivo [19]

Characterization of a retrieved scaffold explant from an animal or human patient involves **weighing, sectioning, staining** and **analyzing the histological features** of the sample. Pathological analysis of the implant site and surrounding tissue using histological staining is another helpful tool in monitoring the inflammatory and immune responses and their effect on scaffold degradation. Hematoxylin and eosin staining is useful for visualize cellular infiltration and scaffold degradation.

Mass loss is one of the most direct characteristics that researchers can use to monitor the in vivo degradation of a scaffold. But the harvested or explanted specimen first needs to have all attached tissue removed. This decellularization process uses a series of freeze/thaw cycles in liquid nitrogen followed by immersion in a 37 °C water bath. When the specimen is clean and free of all adhering tissue, it will be dried and weighed.

Magnetic resonance imaging (MRI) allows tracking of the morphological changes caused by the degradation of the scaffold in-vivo. It is safe, has a good tissue penetration depth and soft tissue contrast. Cells are labelled with ferumoxytol and used MRI to investigate the degradation of the HAp scaffold in a bone defect.

Micro computed tomography (micro-CT) has high resolution and deep penetration and can provide images from the macroscale to the nanoscale. conjugation of gold nanoparticles into a collagen scaffold to enhance the contrast and track the degradation profile of the scaffold.

7. Characterization of drug release scaffolds

Current ways of maintaining therapeutic levels of medications within the bloodstream are limited to repeated administration of drugs either via the oral or parenteral route. This is inconvenient and puts patients at risk of accidental or intentional overdoses. To improve this, it is crucial to develop a delivery system that, once administered, can continue to release drugs in a controlled and sustained manner to achieve safe delivery and maintenance of therapeutically appropriate drug levels long term. [20]

Polymeric micro/nanoparticle or micro/nanofibrous scaffolds have been investigated extensively as carrier vehicles for delivery of therapeutic agents. These scaffolds can deliver drugs to a specific predetermined site while avoiding systemic distribution of their cargo. Compared with their particulate counterparts, micro/nanofibrous scaffolds display several advantages:

(i) their physical structure mimics naturally occurring extracellular matrix (ECM), thus supporting cell adhesion, proliferation, migration and differentiation better than particulate scaffolds.

(ii) they exhibit a higher surface-area/volume ratio and higher interconnected porosity with tunable pore sizes, enabling them to release bio-factors such as proteins or genes and facilitate nutrient and oxygen diffusion as well as waste removal. [20]

Drug loading techniques

Hydrophilic drugs like doxorubicin and chloroquine are effectively encapsulated within hydrophilic polymers including gelatin and PVA, whereas hydrophobic drugs such as paracetamol and ibuprofen (IBU) are better incorporated into and released from hydrophobic polymers like PCL, PLGA and PLA. The long-term release of hydrophilic drugs is, however, more challenging compared with that of hydrophobic drugs.

This is because hydrophilic drugs exhibit poor dispersion within hydrophobic polymers, which usually make up at least part of the carrier vehicle and are highly soluble in the release media (usually water based), leading to a higher risk of burst release. Different drug-loading techniques including surface modification, blending, emulsion and coaxial electrospinning have been employed to encapsulate therapeutic molecules into various scaffolds.[20]

Drug release techniques

The drug, for example ketoprofen or antibiotic, is loaded by immersing precisely weighed amount of scaffolds in drug–ethanol solution in a small glass vial for 48 h at room temperature. Then, the mixture is filtered. The concentration of ketoprofen solution after filtering was determined by using a spectrophotometer at 267 nm. The relative amount of loaded ketoprofen by the scaffolds (A) is calculated from the equation: [21]

$$A = V(C_0 - C_1)/W$$

where V is the volume of ketoprofen solution (mL), C0 is the initial concentration of ketoprofen (mg/mL), C1 is the concentration of ketoprofen solution after adsorption (mg/mL), and W is the weight of the scaffolds (g).

Drug loaded scaffolds are then suspended in 50 mL of phosphate buffered solution at pH 7.4 contained in a glass bottle. This dissolution medium is stirred at 100 rpm in a horizontal laboratory shaker and maintained at 37°C in a water bath. Samples are periodically removed for testing and the volume of each sample is replaced by the same volume of fresh medium. The loss of drug content by doing so at each time point is calculated spectrophotometrically to get the correct drug release profile. [21, 23]

Furthermore, the release of the drug from the investigated scaffolds obeyed quasi-Fickian diffusion mechanism. This mechanism is based on hydrolysis as the polymer is hydrated, swell and then the drug diffuses through the swollen matrix system to the exterior, which ultimately slows down the kinetic release. It is also noted that by increasing drug content in the scaffold (5 to 10 and 20 %) the drug release was increased. This might be due to that higher drug content resulted in higher concentration difference between scaffold and the release medium which cause a higher drug release rate.[22]

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