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**A NATIONAL MEASUREMENT
GOOD PRACTICE GUIDE**

No. 89

Characterisation of
Polymeric Tissue Scaffolds



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Measurement Good Practice Guide No 89

Characterisation of Polymeric Tissue Scaffolds

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Abstract

This Guide describes *current* good measurement practice for obtaining structural information for polymer-based tissue scaffolds and methods for quantifying these data. It covers:

- Imaging techniques, for example, scanning electron microscopy and image analysis.
- Intrusion methods: mercury porosimetry and capillary flow porometry.
- Measurement of sample density and derivation of porosity using the buoyancy method.

Example results are presented to illustrate and compare data generated by the above methods. Although this document focuses on polymer based tissue scaffolds the principles can be applied to other porous materials subject to their properties and the constraints of the techniques.

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Characterisation of Polymeric Tissue Scaffolds

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Foreword

This Good Practice Guide describes methods for assessing the structural characteristics of polymer-based tissue scaffolds.

The information presented in this Guide has been gathered under a project, *MPP4.2 Characterisation of tissue scaffolds*, which formed part of a DTI funded programme of research, *Measurements for Processing and Performance*. The project was carried out by a collaborative team consisting of NPL, the University of Brighton and the IRC in Biomedical Materials, Queen Mary University of London.

This Guide aims to provide guidance to researchers on how to characterise, and therefore optimise the structures of polymer-based tissue scaffolds for tissue engineering applications and to develop robust manufacturing routes. As this topic is still being researched it is currently not possible to be more prescriptive about which techniques to use with particular materials or types of scaffold. The only published standard in this area, ASTM 2450-04, is also a guide to available methods and how they might be used to obtain quantifiable information from tissue scaffolds.

A general familiarity with laboratory operations and use of electron and light microscopes is assumed.

1 Scope

This Guide presents guidance for good practice for determining the structural characteristics of polymer-based tissue scaffolds, i.e. porosity, pores size distribution and permeability. It covers:

- the interpretation of images obtained from a variety of methods including scanning electron
- microscopy.
- the use of intrusion methods: mercury porosimetry and capillary flow porometry.
- the measurement of scaffold density and derivation of porosity using the buoyancy method.

Example results are presented to illustrate the above methods, and to enable comparisons of different techniques. Such comparisons serve to highlight significant differences in the underlying physics between different experimental approaches that directly impact on the perceived structure of the scaffold being studied.

2 Introduction

Porous foams manufactured from a range of polymers including mixtures of polylactic acid, polycaprolactone and hyaluronic acid are being used as temporary housings in which to culture cells [1-3]. These tissue scaffolds are typically relatively stiff materials that degrade over a period of time either *in vitro* or *in vivo*. Many papers have been published on this generic group of tissue scaffolds [1-3] in the search for materials and structures, which can be used with specific cell types to produce new functional tissue. Ideally this new tissue should be identical, both phenotypically and genotypically, to that of the native material from which the cells have been harvested. There are many well recognised issues that remain to be addressed before these objectives can be achieved including the practical problems of providing a suitable culture environment within the scaffold and detecting minor changes in both the short- and long-term behaviour of the cells.

Exactly what a suitable culture environment within a scaffold means is open to debate; it is clear that cells in the core of a developing tissue construct will require an appropriate level of oxygen to prevent hypoxia, a supply of nutrients, removal of waste products and some stability in the local pH. The increase in cell density that occurs over time will increase demand for nutrients and oxygen, and the production of waste. Increased cell density, the build up of extracellular matrix and protein accretion will inevitably lead to a reduction in the diameter or even blockage of pores that will impact on the diffusivity of molecules through the scaffold. Time dependent degradation of the scaffold, that may or may not be enhanced by enzymes produced by the growing cells, adds to the complexity of this situation by altering the local pH, local chemistry and pore size distribution. The kinetics of this process are also affected by the presence of adsorbed proteins and attached cells.

The majority of publications in tissue engineering do not dwell on the structural characteristics of the scaffolds being used in this developing field either in the short- or long-term. Indeed most investigators [4-6] report some average pore size dimensions based on images derived from electron microscopy. These data are usually in the micrometre range and may include some measure of the range of pore sizes, i.e. upper and lower bounds. This lack of detailed data makes it very difficult to optimise the structure of scaffolds, manufacturing processes or indeed to begin to understand the complex perfusion characteristics of a cell-seeded scaffold over time.

The methods outlined in this guide have been used to evaluate the structure of a polycaprolactone tissue scaffold manufactured using the salt leaching method [7]. However the methods described within the text are applicable to scaffolds produced by different methods and materials subject to practical issues that are described herein.

The Guide is written with a multidisciplinary audience in mind.

3 The structure of tissue scaffolds

The practical uses of a tissue scaffold will depend on the geometry of the material, its overall porosity, the size distribution of pores and their interconnectivity. Of these parameters, porosity although most often quoted is actually not a particularly useful quantity as it is only a measure of the total pore volume and does not provide any clue as to how this is distributed through the sample. Moreover the porosity of a given sample can be unduly influenced by defects such as one or more relatively large cavities. These are commonly found in scaffolds produced by processes such as salt leaching.

Porous materials, such as tissue scaffolds can contain three different pore types as shown schematically in Figure 1 [8]:

- Enclosed pores that are isolated within a matrix.
- Open- or through-pores that connect to the outer surface of the scaffold and permit flow of liquid (or gas) from one side of a structure to the other
- Blind-end pores that have contact with an exposed internal or external surface through a *single* orifice.

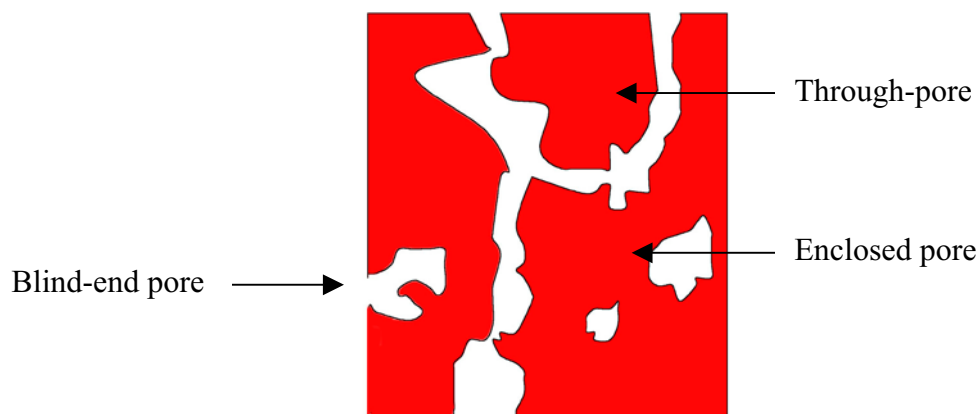


Figure 1: Schematic representation of the different pore types that can exist within a tissue scaffold [after 8]

All of these pores have a role to play in tissue engineering; for example, closed pores can reduce the path length for diffusion of oxygen to the core of the scaffold providing that it can pass through the wall of the scaffold. Through-pores are key to the function of the scaffold providing conduits for cell movement and solute diffusion. Blind-end pores have a less well-defined role although their presence facilitates gaseous diffusion into the structure by reducing

the path length. Whilst it is clear that the presence of these different pore types is advantageous for the performance of the scaffold their presence increases the complexity of characterising the material. Even simple measures of porosity based on density will be subject to increased uncertainty if samples contain different combinations of these pore types. A problem that is exacerbated for tissue scaffolds manufactured from semicrystalline polymers due to the additional difficulty of determining the level of crystallinity within the scaffold and its homogeneity; factors that depend on the manufacturing process.

A variety of techniques are used to manufacture tissue scaffolds including electrospinning, solid freeform fabrication which produce structures that vary enormously in terms of their ‘complexity’, as shown in Figures 2 to 4 and range from well defined regular to irregular geometries. Figure 5. shows a scaffold that has ‘window-like’ apertures appearing in the ‘walls’ of the pores which questions the definitions of what is a pore and what is not.

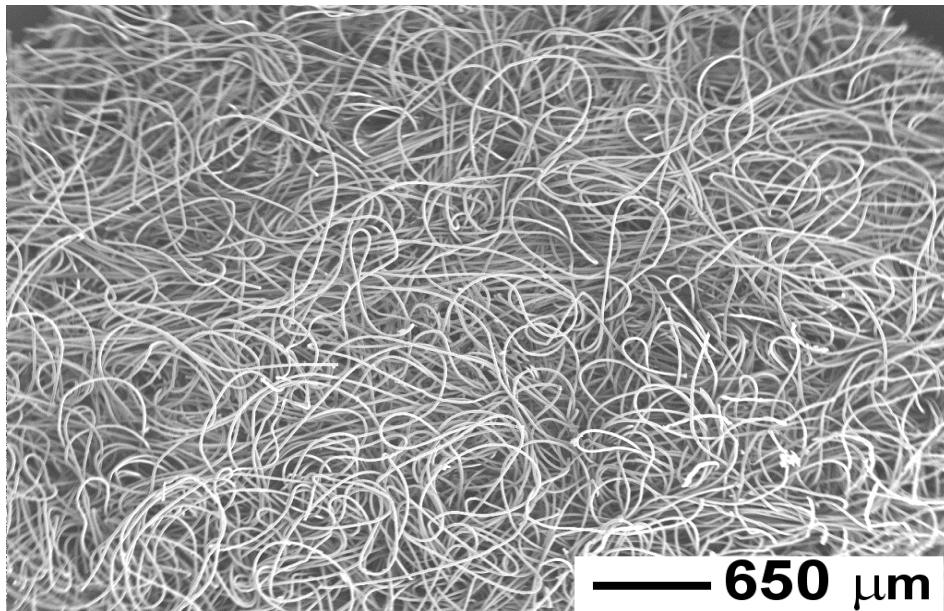


Figure 2: Fibrous meshes can be produced by techniques such as electrospinning.

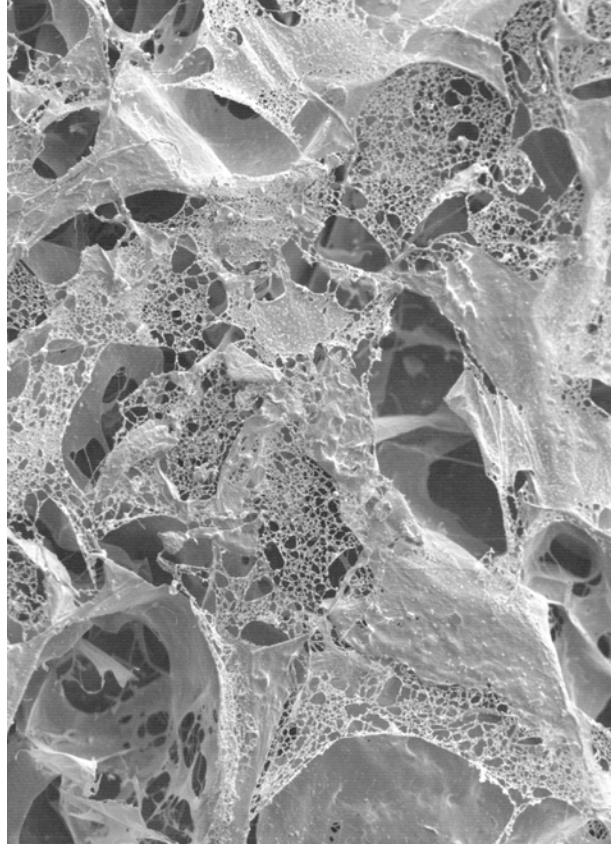


Figure 3: The highly porous structure of a collagen-glycose-amino-glycan matrix (sample prepared by rapid freezing in slush nitrogen)

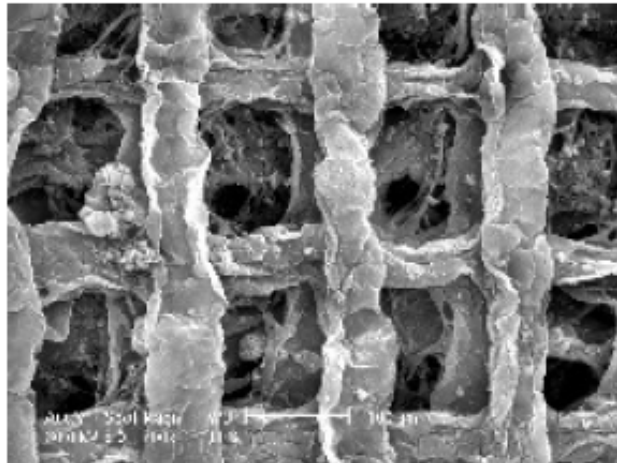


Figure 4: Well-defined scaffolds with controllable geometries can be produced by microfabrication. [9].

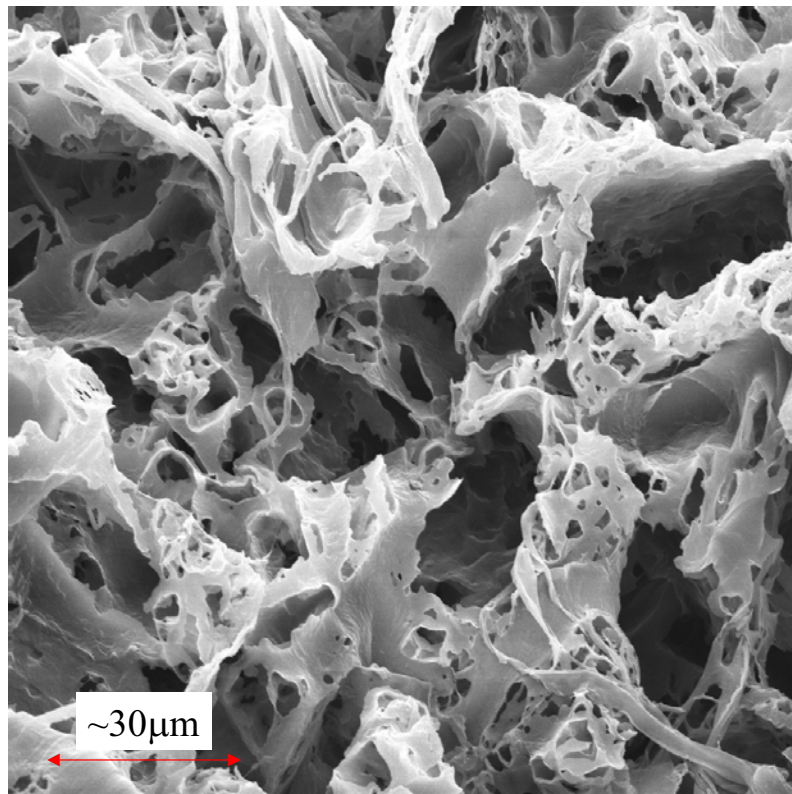


Figure 5: Some scaffold structures are very complicated with window-like apertures appearing in the walls of the pores as shown in this salt-leached polycaprolactone matrix.

4 Techniques for structural evaluation

Table 1 lists some of the techniques that are used to characterise porous materials together with an indication of the information that they provide [10]. The suitability of these different methodologies for investigating the structure of a particular material depends on the size and type of pore as well as other material characteristics e.g. the degree of hydrophobicity or hydrophilicity and the contrast between it and the surrounding medium. The techniques that are discussed in more detail within this guide are highlighted in *italics* within Table 1.

Table 1: Techniques used to investigate different structural aspects of scaffolds; those in *italics* are discussed in detail within this document.

Generic Approach	Generic technique	Information available	Specific Examples
Image Analysis (section 5)	Microscopy	Pore shape, size and size distribution, porosity.	<i>Scanning electron microscopy</i> Optical microscopy Confocal microscopy Optical coherence tomography Optical coherence microscopy
	Micro X-ray computer tomography	Pore shape, size and size distribution, porosity.	
	Magnetic resonance imaging	Pore shape, size and size distribution, porosity.	
Intrusion methods (Section 6)	Porosimetry	Porosity, total pore surface area, pore diameter, pore size distribution	<i>Mercury porosimetry</i>
	Porometry	Median pore diameter (assuming cylindrical geometry), through-pore size distribution, permeability	<i>Capillary flow porometry</i>
Direct measurement (Section 7)	Measurement of density	Porosity, pore volume	<i>Buoyancy method</i>
Molecular probes	Diffusion of markers	Permeability	Cyclic voltammetry Molecular diffusion

5 Image analysis

Image analysis is the most popular approach to studying the structure of tissue scaffolds and for obtaining quantifiable data on pore sizes and the overall level of porosity [36]. The success of this approach is dependent on the way in which the image is obtained, stored and analysed. Care should be taken to ensure that the image is not degraded after capture as a result of controllable factors i.e. storing as a compressed data file. The generic approach to image capture and analysis is shown in Figure 6 and the topics identified are discussed in more detail below.

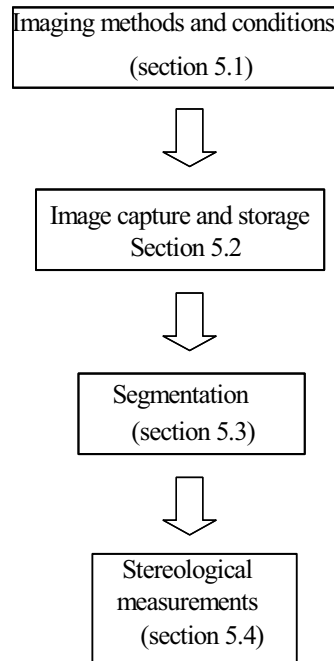


Figure 6: Key stages in image capture, storage and analysis.

5.1 Imaging methods and conditions

A variety of methods can be used to image tissue scaffolds that include:

- Scanning electron microscopy (SEM)
- Transmission electron microscopy (TEM)
- Microcomputer tomography (x-ray) (μ -CT)
- Laser based confocal microscopy
- Optical coherence tomography (OCT)
- Terahertz imaging
- Light microscopy
- Magnetic resonance imaging (MRI)

There have been many books written about the practical use of these methods [11,12,13] and sample preparation, information that is beyond the scope of this guide which focuses on those principles of image analysis that are applicable to a range of techniques. The ‘value’ of the information obtained using these techniques depends on the type of material and the structure of the scaffold. Factors such as structural distortion during sample preparation, instrument resolution and available contrast all have an impact on the quality of the resultant images. Some techniques such as conventional light microscopy and scanning electron microscopy produce 2-dimensional, (2-D) representations of 3-dimensional structures, (3-D)

that can be difficult to interpret, section 5.4. Laser confocal microscopy and optical coherence tomography (OCT) are used to recreate a 3-D virtual image of a scaffold by assembling a series of 2-D images obtained at different locations within the sample. However, these techniques have limited penetration into the sample due to loss of light through scattering. To some extent this limitation can be overcome by flooding the structure with a fluid that has a comparable refractive index to the scaffold material, a process termed index matching which enhances penetration. Further information on the factors that influence wide field and confocal microscopy can be found in references [14,15,16] and in reference [17] for optical coherence microscopy.

Terahertz imaging, MRI and micro-computer tomography can be used to obtain 3-D images of much larger blocks of tissue scaffold although the resolution is limited and, depending on the instrument, ranges from about 1 to 10 μm . This relatively low resolution is suitable for imaging large pores i.e. in excess of 100 μm but will obscure the important fine structure of the scaffold. There is also a limitation on how well a 2-D or 3-D image represents the physical artefact, which is particular to the imaging method used and depends on such factors as spatial resolution and dynamic range, image contrast and the signal to noise ratio.

The reconstruction of a 3-D image from 2-D images obtained from a physical series of 2-D slices requires considerably more effort in sample preparation, image collection and analysis than re-assembly of virtual sections produced by techniques such as laser confocal microscopy. Laser confocal microscopy and OCT focus on a series of planes within the sample located at different depths. Assembly of these virtual images avoids any complications due to sample distortion caused by microtoming and issues with registration¹ although the penetration depth is, as previously mentioned, limited due to scattering. Both confocal microscopy and OCT have penetration depths of about 100 μm depending on the structure of the scaffold and the wavelength of the laser used. Increasing the wavelength of the incident radiation improves penetration but at the expense of resolution. It is possible to collect a series of 2-D virtual slices in a region of the sample to the point at which scattering losses become too high. This material can then be removed by microtoming and the process repeated to build up a 3-D image through a combination of physical and virtual slices. This approach becomes both labour intensive and is subject to errors as reconstruction of the virtual 3-D structure involves assembly of both the virtual and physical slices with consequent concerns over registration and depth accuracy.

5.2 Image capture and storage

¹ Image registration refers to the process of orienting a series of images perhaps captured at different orientations or at different times into a common set of coordinates which greatly simplifies their analysis.

Images of tissue scaffolds will almost certainly be captured, stored and analysed in digital form. Care should be taken to ensure that the process of image capture and storage preserves as much information as possible. Image capture generally involves an analogue to digital (A/D) conversion of an image into an electrical signal and hence a bit-map. This process involves quantifying the greyscale value of the image at all points across its surface. Typically images will comprise of brightness transitions between dark and light. The cycle rate between these regions is referred to as the spatial frequency of the image. The spatial frequency is relatively constant over the viewing area for images that have large blocks of comparable tonality, but for many images this is not so. The Nyquist criterion states that the sampling distance should be at least twice the highest spatial frequency observed in the image. Failure to comply with this requirement will result in a loss of fine detail. Note that the Nyquist criterion applies to differences in brightness and not to the spatial resolution of pixels that are covered by Shannon's sampling theorem².

5.2.1 Analogue to digital conversion

Digitization of analogue signals can be done at 8-bit or 16-bit conversion³. 16-bit conversion produces a superior image with a broader range of tonalities and is worthwhile if extensive image enhancement is required or if there is a high degree of contrast within the image. However the file size for a 16-bit image is much larger than for a 8-bit image and for most purposes, 8-bit conversion is perfectly suitable. It should also be recognised that some image analysis programmes may have limited options for manipulating 16-bit files. 8-bit conversions in monochrome have 256 grey levels compared to 65,536 for a 16-bit conversion; in both cases level 0 corresponds to pure black and the highest level, 255 to pure white. Colour images are usually represented as combinations of red, green and blue (RGB), each of which is represented as 256 levels in an 8-bit conversion. The number of colours available is 256^3 from an 8-bit conversion that increases to billions for the 16-bit equivalent.

5.2.2 Minimum detectable dimensions

It is important to record the minimum detectable distance for all magnifications. In 2-D the minimum detectable distance corresponds to the dimensions of a single pixel and to that of a voxel in 3-D space. Measurements at sub-pixel levels are possible through extrapolation but are obviously subject to higher uncertainties.

² Shannon's sampling theorem states that the sampling interval i.e. distance between the centres of two adjacent pixels would be less than 0.5 the size of the smallest desired detectable feature.

³ The greyscale information per pixel is represented in terms of either 8-bits or 16-bits of information

5.2.3 File storage

It is tempting to store digital files as compressed files e.g. JPEG or GIF, however this will result in loss of data to an extent that depends on the amount of compression used. This data loss occurs in the high frequency components of the Fourier spectrum leading to blurring of edges in monochrome images and smeared colour intensity profiles for RGB images. A useful discussion regarding file decompression and decompression can be found in reference [18].

5.2.4 A method for improving weak signal strength

There will be cases where the contrast between the scaffold and the surrounding medium will be low. This can cause the image to appear noisy due to random fluctuations in signal intensity in the digitised image. The strength of the signal compared to the noise level, the s/n ratio can be improved by averaging a number of frames, N . The invariant signal strength increases with respect to N whilst the random noise scales by the square root of N leading to an improvement in s/n of $N/\sqrt{N}$.

5.2.5 Ensuring constant illumination over the sample

Image analysis software is based on the differences in grey scale intensity or shade of colour that the image consists of and therefore any unevenness in illumination over the sample area can lead to misinterpretation. Uneven illumination can occur due to the source itself, to a non-linear response of the detector or poor alignment of the sample. The effects of non-linear illumination can be removed by subtracting a background captured in the absence of a sample or by adjusting the greyscale uniformity in image processing software packages such as Photoshop.

5.2.6 Feature enhancement

Techniques are available for enhancing the features of interest within an image, for example, mask mode radiography. In this technique an x-ray image is obtained before and after the addition of a contrast-enhancing agent. Subtraction of the first image from the second will highlight the key features.

Images can also be sharpened, smoothed, de-blurred or restored by applying a filter to the Fourier transform of an image. The Fourier transformation of image data converts spatial information into a frequency spectrum, fine detail will be represented by higher frequencies and vice versa. Removing high or low frequency components of this frequency distribution will

remove spatial features. The effects of filtration become apparent after converting the frequency domain representation of the image back into real space. This approach is commonly used in signal processing applications.

5.3 Segmentation or thresholding

A 256 greyscale monochrome image will have tonalities that range from pure black (greyscale value 0) to pure white (greyscale value 255). In an ideal situation level 0 regions will correspond to the pores within the scaffold and the nearly white regions approaching level 255 to the walls. In practice images show a range of tonalities, Figure 7. The challenge of segmentation or thresholding is to segment this type of greyscale image into regions associated with the pores and walls respectively. The intensity of the greyscale image shown in Figure 8 ranges from dark grey pixels to light grey pixels depending on whether a pore or wall edge is being viewed. The contrast in an image such as this can be artificially enhanced by expanding the histogram so that it does cover the full gamut from level 0 to level 255. An inevitable consequence of this action is that the introduction of periodic gaps in the histogram which although are cosmetically unsightly don't influence subsequent analyses.

There are usually a number of options available in image analysis software for finding the threshold that range from fully automatic to a manual 'by eye' approach. The latter method, although subjective is actually a very good approach even between individuals. Selecting the threshold manually allows the user to explore different options with some software packages providing feedback as to which areas of the image are affected by the changes. This feedback usually takes the form of a coloured mask which overlays the image being analyzed. Programmes such as ImageJ [19] allows the user to position the mask over the void regions or the walls depending on their preference. The objective methodologies used in automated routines are based in a statistical analysis of the histogram [20]. Interpreting the histogram in terms of two overlapping normal distributions associated with the pores and walls respectively. Otsu [21] has proposed an alternative procedure that doesn't rely on probabilistic density distributions but utilises the variance as a measure of homogeneity. The threshold is found by minimising the variance in pixels associated with the pores and walls respectively. Other approaches to thresholding are available that use changes in brightness or texture to locate edges, for example, the marching cubes algorithm [22].

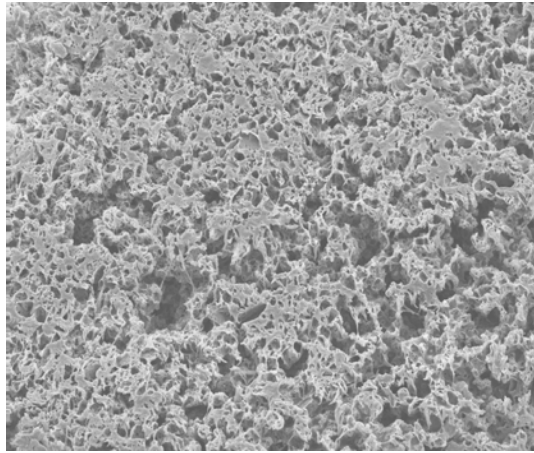


Figure 7: A low contrast micrograph of a polycaprolactone scaffold produced by salt leaching

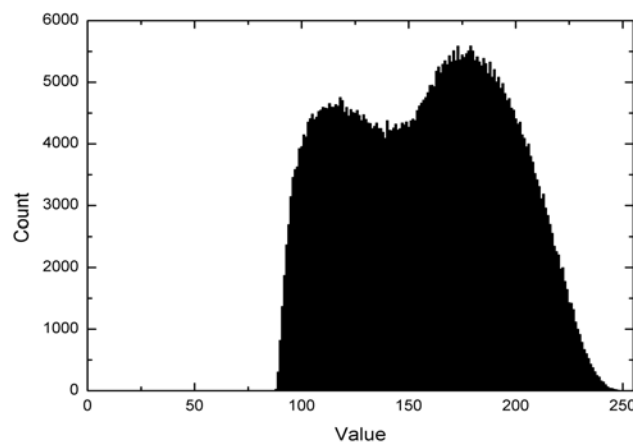


Figure 8: A bimodal histogram of grayscale intensities corresponding to the image shown in Figure 7 has a limited gamut mirroring the lack of contrast in the original image

The magnification used to obtain the image can affect thresholding particularly if it carried out manually. Walls can appear to be better defined in low magnification images due to the increase in the minimum detectable distance.

5.4 Stereological Measurements

Once the threshold greyscale value has been defined the greyscale image is reduced to a binary black and white image. Tonalities between pure black and the threshold are reduced to black, level 0 and those between the threshold and pure white are converted to level 255 (pure white). The binary image which is now unambiguously divided into pores and walls, can be analysed in

Table 2. Parameters that can be used to quantify pore shape and size (based on ASTM 1877 [23]).

Aspect of pore morphology	Parameter	Definition
Pore shape	Aspect ratio (AR)	The aspect ratio of the major diameter, $d_{\max}$ i.e. the longest line that can be drawn between two points on an outline to the minor diameter, $d_{\min}$. The minor diameter is defined as the longest line perpendicular to the major diameter that connects two points on the outline. An aspect ratio can also be derived from the ratio of orthogonal Feret diameters, see below.
	Elongation (E)	The elongation is similar to the aspect ratio and is defined as the ration of the pore length to the average particle width. Elongation is particularly useful for characterizing long pores where the major axis line doesn't stay within the pore boundaries.
	Roundness (R)	The roundness, R , provides a measure of the circularity of a pore. A perfect circle has a roundness of 1. R is calculated from the area of the pore (A), and the maximum diameter ($d_{\max}$) according to $R=(4A)/(\pi d_{\max}^2)$
	Form Factor (FF)	Pore circularity can also be assessed using the form factor, FF , that uses the perimeter of the pore outline, p , rather than $d_{\max}$, i.e. $FF=4\pi A/p^3$ (A = area, p = perimeter)
Pore size	Equivalent circle diameter (ECD)	The area of an irregularly shaped pore can be expressed in terms of an equivalent circle diameter, ECD , i.e. $ECD=(4A/\pi)^{1/2}$. Correspondence between the ECD and the actual pore diameter improves with increasing roundness.
	Feret diameter	The Feret diameter is defined as the mean distance between pairs of parallel tangents to the periphery of a pore. This approach is particularly valuable for analysing round or rectangular-shaped pores.

terms of the ratio of pore area (or volume) to sample area (or volume), to obtain the porosity. The dimensions and shape of the pores can also be analysed to produce a set of quantifiable measures that represent the structure. Various approaches are used to quantify shape and structure, those used for classifying particles that can be applied to pores, are given in Table 2. Drawing a series of lines from one boundary to another, i.e. chords can, and are, used to provide estimates of particle sizes; the distribution of chord lengths is a measure of the particle

5.4.1 Alternative methodologies for analysing irregularly shaped pores

An approach to analysing irregularly shaped pores is to fill the pore volume using spheres of different diameters in a virtual environment [24]. Clearly the spheres with largest diameters be fill the bulk of the pore volume, smaller spheres filling interspherical spaces and smaller volume regions of the pore. This distribution of sphere diameters provides a measure of the mean pore diameter. The loci of the largest sphere centres produce a skeleton that represents the path of a pore through the scaffold. This skeleton can be used to assess pore interconnectivity and tortuosity.

The interconnectivity within a porous structure is a measure of the connectivity of pores, low levels of connectivity are likely to be unsuitable for most tissue engineering applications due to constraints on solute movement. Though this influence is of course structure and tortuosity dependent, for large pore diameters lack of connectivity will not be an issue and for those scaffolds that are designed to guide tissue growth any interconnectivity is undesirable.

Tortuosity is a measure of the path length that a tracer particle will take as it passes through a scaffold from one point to another. Tortuosity is a key factor in tissue scaffold performance but rarely quoted in the literature. There are many methods available for calculating a measure of tortuosity, an example of which is to use the ‘inflection count metric’, ICM. This approach adds up the number of inflections of a 3-D Frenet frame representation of a pore connecting two points and multiplies this number by the path length [37] (the Frenet frame is determined by fitting tangents to the image of the pore walls). The resultant product when divided by the actual distance between the two end-points is a measure of tortuosity. An alternative approach is to sum the curvature of the skeletonized representation of the pore and divide this value by the path length [37].

5.4.2 Determination of porosity

The porosity in 2-D or 3-D is given by the volume fraction, V_p of pixels or voxels, PP_i that represent pores with respect to the number contained in the whole image, PP_t . In 2-D this is given by

$$V_p = A_p = A_p/A_t = PP_i/PP_t$$

In both cases there is some uncertainty in the porosity estimate due to the resolution of the image and errors associated with segmentation that is difficult to quantify. Some software packages require the user to define a lower limit for the number of pixels that constitute a pore. This, whilst acting as a crude filter adds to the uncertainty within the estimated porosity.

Estimates of pore size and the overall level of porosity will be affected by factors that include the homogeneity of the size and shape of the pores and their orientation with respect to the image as shown in Figure 9. Such issues are less important for random pore sizes and shapes where orientation effects are averaged out.

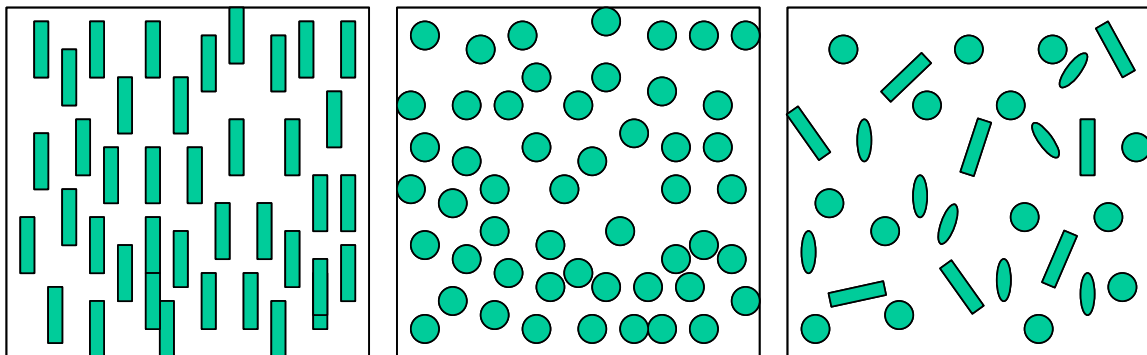


Figure 9: The pore morphology, size and porosity of oriented samples can be very sensitive to the image orientation as shown above for rod-like pores. This is less important for irregular or randomly oriented structures.

Care should be taken to ensure that images obtained from different samples within a series are captured at the same magnification. Guidance regarding the optimal magnification for different pore sizes is given in Table 3, of course after the image has been captured it can be viewed electronically at any magnification.

Table 3: Recommended magnifications for imaging pores. This information is based on particle analysis methods described in ASTM 1877 [23].

Magnification	Pore size range (µm)
10,000	0.1 to 1.0
1000	1 to 10
100	10 to 100

6 Intrusion methods

Intrusion methods rely on temporarily filling the scaffold with a fluid. In mercury porosimetry the pressure required to force mercury into the structure provides a measure of the pore sizes and density of pores (Section 6.2). In gas flow porometry, comparable information is obtained by monitoring the time dependent pressure required to displace fluid from a liquid filled material (section 6.1) which also produces a measure of the permeability of the scaffold. Both mercury porosimetry and gas flow porometry are able to detect through pores and the former method is also sensitive to blind-end pores. Neither method is sensitive to the presence of closed pores.

6.1 Capillary flow porometry

Information on the distribution of pore diameters can be obtained by using liquid extrusion techniques, such as capillary flow porometry. This technique involves flooding the sample with a wetting fluid that fills all the accessible pores. A non-reactive gas under increasing pressure is then used to displace this fluid from the sample. The amount of pressure required for this process depends on the size and size distribution of pores. The wetting fluid is chosen such that its surface free energy with the sample is less than that of the sample with the gas. This enables the gas to displace the wetting fluid, replacing the liquid-sample interface by a high free energy gas-sample interface, i.e.

$$p = (\gamma_{s/g} - \gamma_{s/l}) dS / dV \quad (1)$$

where p is the differential pressure, dS/dV is the rate of increase in the surface/gas interface. $\gamma_{s/g}$ and $\gamma_{s/l}$ refer to the surface energy between the sample and gas and sample and liquid respectively. In practice the gas pressure must exceed the capillary forces responsible for liquid uptake into the sample.

The difference in surface free energy between the liquid- and gas-solid interfaces is related to the surface tension, γ and contact angle, θ of the wetting fluid according to

$$(\gamma_{s/g} - \gamma_{s/l}) = \gamma \cos \theta \quad (2)$$

And hence from (1) and (2) the gas pressure required to displace the wetting fluid is

$$p = \gamma \cos \theta (dS / dV) \quad (3)$$

Pores typically have an irregular cross-section along their length that can vary both in shape and in area. The change in surface area per small increment of volume dV is related to a change in an equivalent pore diameter, i.e.

$$p = \frac{4\gamma \cos \theta}{D} \quad (4)$$

the pressure p required to displace fluid depends on the contact angle (θ), pore diameter (D) and surface energy (γ).

From equation (4) the pressure required to displace the wetting fluid from pores is inversely proportional to their diameter. The lowest pressure required to initially displace fluid from a sample corresponds to the bubble point and equates to the largest pore diameter. Measurement of the volume of displaced fluid provides a direct measure of pore volume as a function of gas pressure. In practice [25,26] the pressure and gas flow rates through wet and dry samples are determined to give ‘wet’ and ‘dry’ curves, Figure 10. The dry curve provides a measure of permeability that is discussed in more detail below. The half-dry curve is calculated from the measured dry curve to give half the flow rate through a dry sample at any given differential pressure. The intersection of the wet curve with the half-dry curve provides the mean flow pressure and hence the mean flow pore diameter. The pore size distribution can be calculated from the difference between the wet and dry curves. The bubble point that occurs at a pressure of 0.81 kPa, corresponding to a maximum pore diameter of 54.8 μm for the example shown in Figure 10. The median flow pressure of 7.6 kPa equates to a median pore diameter of 5.9 μm .

The median flow pore diameter can also be obtained from a plot of cumulative flow versus pore size as shown in Figure 11. The median flow pore diameter is that which corresponds to 50% cumulative flow. In practice the median flow pore diameter states that 50% of the gas will flow through pores larger than 5.9 μm and 50% will flow through smaller pores

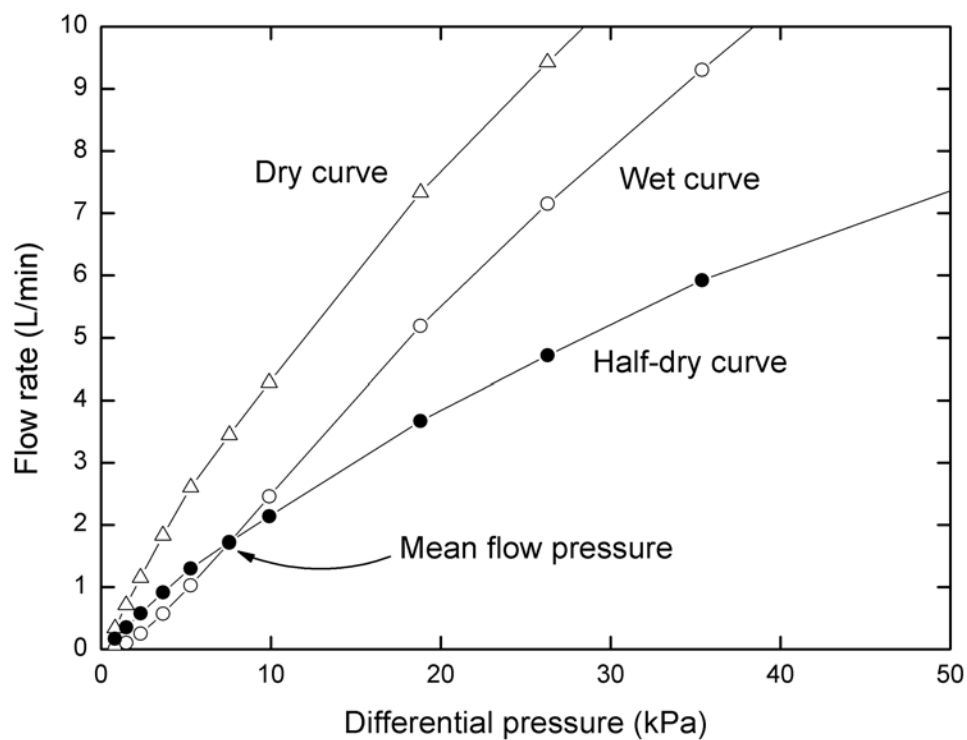


Figure 10: Flow versus pressure for a wet sample (○) and dry sample (△). The $\frac{1}{2}$ dry curve is calculated from the dry curve and gives 50% of the flow for the same differential pressure (●).

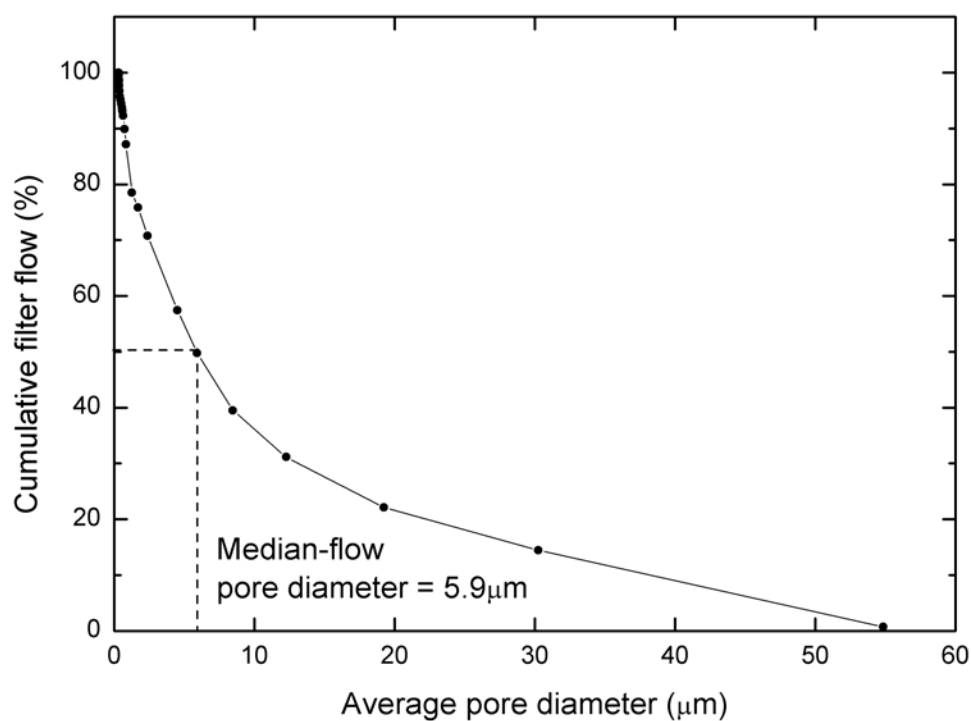


Figure 11: Cumulative-filter flow pore size distribution.

The underlying physics of this approach dictates that the method is sensitive to the *narrowest* point along a pore as shown schematically in Figure 12. Therefore the calculated pore diameters refer to a distribution of constriction points and are therefore usually smaller, by up to an order of magnitude, than pore sizes obtained by other methods.

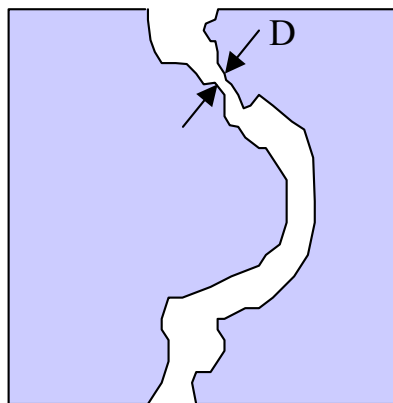


Figure 12: Capillary flow porometry measures the bottle-necks (minimum diameter along a pore)

Data provided by this technique provide an indication of the scaffolds accessibility to cell migration. The measured pore size distributions highlight the minimum pore diameters. Very small bottlenecks are not conducive to cell migration and are likely to have a negative impact on solute diffusion.

A measure of the permeability of the scaffold can be obtained by monitoring the flow of air through the sample as a function of an applied pressure gradient. According to Darcy's Law, the rate, Q at which air flows through a sample of thickness, L and cross sectional area, A for a range of differential pressures, ΔP provides a measure of permeability, k (eq. 5)

$$Q = \frac{kA\Delta P}{L} \quad (5)$$

6.2 Mercury Porosimetry

Mercury porosimetry (or mercury intrusion porosimetry) is a similar technique to gas flow porometry except that the pressure monitored is that required to force non-wetting mercury into a sample under vacuum. The gradual increase in pressure necessary to flood the sample is related to the pore diameter via the Washburn equation (equation 6).

$$D = \frac{- (4\gamma \cos \theta)}{p} \quad (6)$$

where D is the diameter of the pore at a particular differential pressure P , θ is the surface tension of mercury and γ is the contact angle of mercury with the material.

Mercury porosimetry although a relatively simple and quick technique has its limitations [27-30] in that it:

- Cannot detect closed pores
- Cannot measure small mesopores ($D < 7$ nm) due to lack of penetration by the mercury.
- Cannot measure very large pores (D in excess of several hundred micrometres) as the mercury penetrates the structure before measurements can be made.
- Is sensitive to ink-bottle pores⁴
- Can detect through- and blind-end pores but cannot distinguish between them

The technique assumes that the pore geometry is regular and that they are interconnected. Two parameters, the skewness and kurtosis can be used to quantify the distribution of pore sizes. Skew is a measure of the symmetry of a distribution of data. If the median and mean are equal then the data will be symmetrical about this point and the skew will be zero. Negative skew occurs when the mean is less than the median and data are asymmetric with a bias towards lower values, the converse is true for positive skew. The magnitude of the skew is a direct measure of asymmetry. Kurtosis is a measure of the relative concentration of values in the centre of the distribution compared with those in the tails, a normal distribution has a kurtosis of 3. Higher kurtosis values imply a sharper distribution than expected and vice versa.

Values of skew and kurtosis should be assessed by comparing the repeatability of results on the same sample before using these quantities to determine the in-homogeneity within a sample, batch-to-batch variations or structural changes resulting from modifications to the manufacturing route.

7 Determination of porosity from density measurements

The density of a material is defined as mass/volume therefore a measure of porosity can be obtained by determining this quantity providing the solid density of the bulk material is known.

⁴ Ink-bottle pores are large pores with narrow entrances which consequently fill at higher pressures than would be expected causing some distortion within the pore size distribution. They can be detected by examining the hysteresis between filling and draining the scaffold.

The mass of the material can be easily measured to a high level of accuracy. However, this same degree of accuracy is much more difficult to achieve when measuring the volume of a material, particularly for irregularly shaped samples. This issue can be overcome by employing Archimedes Principle, which states that the volume of liquid displaced by an object completely immersed in it is equal to the volume of the object. This relationship can be used to determine the density of an unknown material, either by measuring the decrease in weight of a body due to buoyancy (buoyancy method) or by measuring the weight of the fluid or gas displaced (e.g. helium pycnometry) [12].

7.1.1 Determination of density and porosity

The buoyancy method relies on weighing the sample in both air, $m_{(a)}$ and liquid, $m_{(fl)}$, e.g. water of density ρ_{fl} . The mass of liquid displaced due to the volume of the sample is given by $m_{fl} = m_{(a)} - m_{(fl)}$ and the apparent solid density⁵, ρ_s , is then defined by equation 7 [31]:

$$\rho_s = \frac{m_{(a)}}{m_{(a)} - m_{(fl)}} \rho_{fl} \quad (7)$$

Thus the general equation between the density and mass of a liquid in which the sample is immersed and a solid object that has an unknown volume, is:

$$\rho_s = \frac{m_s}{m_{fl}} \rho_{fl} \quad (8)$$

This relationship enables the unknown apparent density of the solid object to be determined. If the assumption is made that the scaffold walls are free from enclosed pores then the apparent density is a true density⁶.

The apparent porosity⁷, π_a , can be determined by measuring the mass of a sample soaked in the fluid (m_{soaked}) in air according to [31]:

$$\pi_a = \frac{m_{soaked} - m_{(a)}}{m_{soaked} - m_{(fl)}} \times 100 \quad (9)$$

⁵ Apparent solid density is the ratio of the mass of the dry material to its apparent solid volume. Apparent solid volume is the sum of the volume of closed pores and true volume where the true volume is that of a body occupied by solid material excluding all forms of porosity [13]

⁶ True density is the ratio of the mass of dry material to its true volume.

⁷ Apparent porosity = ratio of total volume of open pores in a porous body to its bulk volume [13].

For moderately hydrophobic materials the consistency of repeat measurements can be significantly improved by exposing the sample to an extended period of soaking and agitation, if water is used as the wetting fluid. This can be monitored by repeatedly measuring m_{soaked} until it becomes stable.

Estimates of porosity obtained using this method assume that the pores within the structure are fully wetted out and that the proportion of enclosed or blind-end pores is negligible: this assumption can be checked by determining the apparent density of the solid material. A significant disparity i.e. lower than expected value for the density of the solid material is indicative of either a significant percentage of voids or insufficient wetting of the pores or some combination of the two which increases the buoyancy of the scaffold reducing m_{fl} . Helium pycnometry can be used to distinguish between these two effects. This technique relies on measuring the material volume within the scaffold from a change in helium pressure due to the presence of the scaffold in a known volume of gas [32-34]. Helium is able to fully penetrate the porous structure of the scaffold without any encountering any problems with surface tension.

Helium pycnometry can also be used to obtain an estimate of porosity providing that the porous sample is sealed to prevent gas penetration. There are experimental problems that need to be addressed to ensure that the seal is effective and that it doesn't significantly alter the volume or weight of the sample which are beyond the scope of this guide.

8 Determination of pore size distributions

The pore size distribution is key to its performance influencing solute transport, cell migration and scaffold degradation [35] but not easy to determine, especially for complex structures where the definition of a pore is open to debate. The analysis of 2-D images obtained from scanning electron microscopy relies on accurate thresholding i.e. on being able to differentiate between a cavity and a wall. This procedure is open to ambiguity especially for high magnification images, as the greyscale intensity of material located at the base of a pore can be comparable to that at its rim. This situation improves significantly using lower magnification images that also improves the statistical significance of the data. However resolving the pore size distribution still depends on being able to define a clear continuous edge around a pore in order to map its size. This process for the complex scaffolds studied here is open to substantial error. On this basis, generating a pore size distribution based on the analysis of images of complex structures is not to be recommended.

Figure 13 compares the pore size distributions of the scaffold computed from the intrusive techniques of capillary flow porosimetry and mercury porometry. From this figure it is

apparent that the range of pore sizes derived from capillary flow porometry occurs over a smaller length scale than those based on mercury porosimetry data. This difference is expected since underlying physics of the former technique is sensitive to the bottleneck regions of the pores as shown in Figure 14. The pore diameters based on mercury porosimetry are based on a range of diameters that all contribute to the pressure –time curve from which the pore size distribution is obtained as shown in Figure 14.

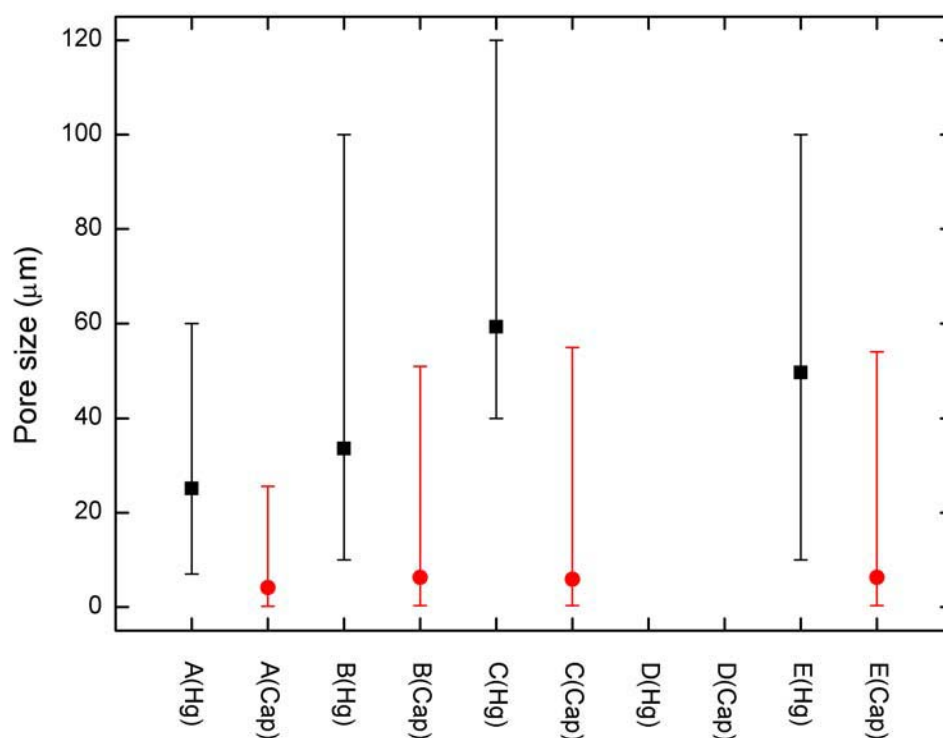


Figure 13: Comparison between scaffolds pore distributions obtained via different measurement techniques for a complex polycaprolactone scaffold manufactured by salt leaching, (cap=capillary flow porometry, Hg=mercury porosimetry, ■ = median value, ● = median value). Intrusion methods were not suitable to characterize sample D due to its low permeability.

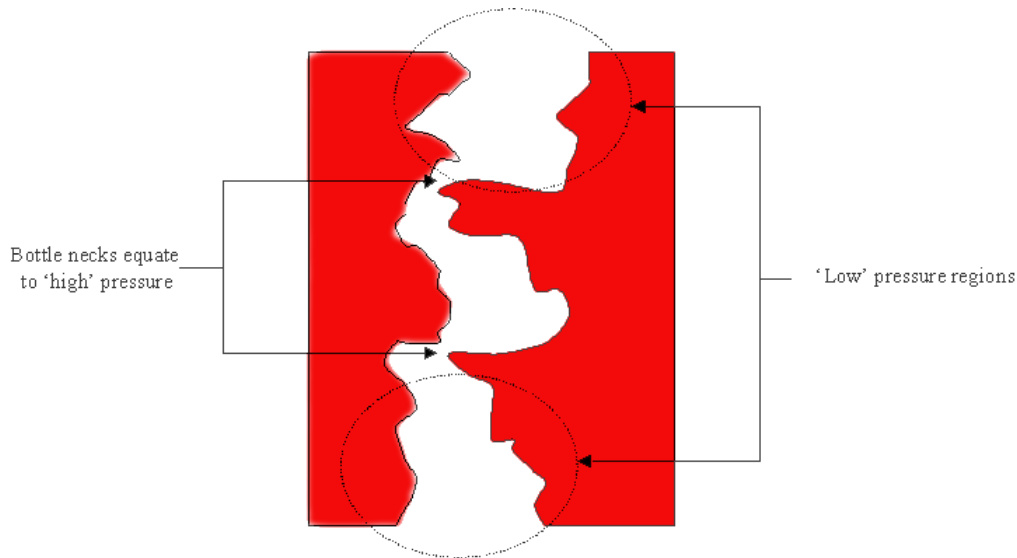


Figure 14: A non-uniform pressure is required to fill an irregularly shaped pore that will contribute to the pore size distribution in mercury porosimetry. Gas flow porometry will be sensitive to the bottle-neck regions.

9 Conclusions

Measures of porosity although commonly quoted actually have little practical value in quantifying the structure of tissue scaffolds since:

- The measure is particularly sensitive to the presence of voids in the sample produced during manufacture.
- Porosities derived from mercury porosimetry, capillary flow porometry or from density measurements are insensitive to closed pores.
- Porosities based on gas flow porometry measurements are not sensitive to blind-end pores.
- Porosity as a parameter is independent of pore size, shape and density.

The distribution of pore sizes can be obtained from both mercury porosimetry and capillary flow porometry as well as image analysis. The former methods produce distributions that are representations of the actual scaffold structure that reflect the limitations of the underlying physics of the techniques. For this reason it is very difficult to compare pore size distributions for complex structures, such as particulate-leached tissue scaffolds obtained using different experimental approaches.

Whilst capillary flow porometry, mercury porosimetry and image analysis can and are used to obtain structural information from a scaffold it must be recognised that the actual range of pore sizes will extend beyond the length scales accessible by these methods [27]. Issues regarding uncertainties and resolution should also be considered although they have not been addressed

in this guide.

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11 Appendix 1: Terminology

The following definitions are taken from an ASTM standard on the physical characterisation of tissue scaffolds [27] and image analysis [35]

Blind (end) pore, n – a pore that is in contact with an exposed internal or external surface through a single orifice smaller than the pore's depth.

Closed cell, n – a void isolated within a solid, lacking any connectivity with an external surface.
Synonym: *closed pore*

Permeability, n – a measure of fluid, particle, or gas flow through an open pore structure.

Pixel, n – 2-dimensional picture element.

Polymer, n – a long chain molecule composed of monomers including both natural and synthetic materials, e.g. collagen, polycaprolactone.

Pore, n – a liquid (fluid or gas) filled externally connecting channel, void, or open space within an otherwise solid or gelatinous material (e.g. textile meshes composed of many or single fibers (textile based scaffolds), open cell foams, (hydrogels). Synonyms: *open pore, through pore*.

Porogen, n – a material used to create pores within an inherently solid material. For example, a polymer dissolved in an organic solvent is poured over a water-soluble powder. After evaporation of the solvent, the porogen is leached out, usually by water, to leave a porous structure. The percentage of porogen needs to be high enough to ensure that all the pores are interconnected.

Porometry, n – the determination of the distribution of open pore diameters relative to the direction of fluid flow by the displacement of a non-volatile wetting fluid as a function of pressure.

Porosimetry, n – the determination of the pore volume and pore size distribution through the use of a non-wetting liquid (typically mercury) intrusion into a porous material as a function of pressure.

Porosity, n – property of a solid which contains an inherent or induced network of channels and open spaces. Porosity can be determined by measuring the ratio of pore (void) volume to the apparent (total) volume of a porous material and is commonly expressed as a percentage.

Roundness (R), n – a measure of how closely an object represents a circle.

Scaffold, n – a support, delivery vehicle, or matrix for facilitating the migration, binding, or transport of cells or bioactive molecules used to replace, repair or regenerate tissues.

Segmentation, n – a methodology for distinguishing different regions within a tissue scaffold image, e.g. pores and walls.

Spherical, adj- a particle with a generally spherical shape that appears round in a photograph (F1877).

Threshold, n – isolation of a range of grayscale values exhibited by one constituent within the image.

Through pores, n – an inherent or induced network of voids or channels that permit flow of liquid from one side of the structure to the other.

Tortuosity, n – a measure of the mean free path length of through pores relative to the sample thickness. Alternative definition: The squared ratio of the mean free path to the minimum possible path length.

Voxel, - 3-dimensional picture element

