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Biomaterials and scaffolds for tissue engineering

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Abstract
Every day thousands of surgical procedures are performed to replace or repair tissue that has been damaged through disease or trauma. The developing field of tissue engineering (TE) aims to regenerate damaged tissues by combining cells from the body with highly porous scaffold biomaterials, which act as templates for tissue regeneration, to guide the growth of new tissue. This article describes the functional requirements, and types, of materials used in developing state of the art of scaffolds for tissue engineering applications. Furthermore, it describes the challenges and where future research and direction is required in this rapidly advancing field.

Overview
Disease, injury and trauma can lead to damage and degeneration of tissues in the human body, which necessitates treatments to facilitate their repair, replacement or regeneration. Treatment typically focuses on transplanting tissue from one site to another in the same patient (an autograft) or from one individual to another (a transplant or allograft). While these treatments have been revolutionary and lifesaving, major problems exist with both techniques. Harvesting autografts is expensive, painful, constrained by anatomical limitations and associated with donor-site morbidity due to infection and hematoma. Similarly, allografts and transplants also have serious constraints due to problems with accessing enough tissue for all of the patients who require them and the fact that there are risks of rejection by the patient’s immune system and the possibility of introducing infection or disease from the donor to the patient. Alternatively, the field of tissue engineering (a phrase that is interchangeably used with regenerative medicine) aims to regenerate damaged tissues, instead of replacing them, by developing biological substitutes that restore, maintain or improve tissue function.

The term ‘tissue engineering’ was officially coined at a National Science Foundation workshop in 1988 to mean ‘the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function’. However, while the field of tissue engineering may be relatively new, the idea of replacing tissue with another goes as far back as the 16th century. Gasparo Tagliacozzi (1546-99), Professor of Surgery and Anatomy at the University of Bologna described a nose replacement that he had constructed from a forearm flap in his work ‘De Custorum Chirurigia per Insitionem’ (The Surgery of Defects by Implantation) which was published in 1597. The field of tissue engineering is highly multidisciplinary and draws on experts from clinical medicine, mechanical engineering, materials science, genetics, and related disciplines from both engineering and the life sciences. The field relies extensively on the use of porous 3D scaffolds to provide the appropriate environment for the regeneration of tissues and organs. These scaffolds essentially act as a template for tissue formation and are typically seeded with cells and occasionally growth factors, or subjected to
biophysical stimuli in the form of a bioreactor; a device or system which applies different types of mechanical or chemical stimuli to cells\textsuperscript{4}. These cell-seeded scaffolds are either cultured \textit{in vitro} to synthesize tissues which can then be implanted into an injured site, or are implanted directly into the injured site, using the body’s own systems, where regeneration of tissues or organs is induced \textit{in vivo}. This combination of cells, signals and scaffold is often referred to as a tissue engineering triad (Fig. 1). In this review, the term 'tissue engineered construct' is used to identify scaffolds which have undergone extensive \textit{in vitro} culture prior to implantation. The term scaffold refers to the 3D biomaterial before cells have been added (\textit{in vitro or in vivo}).

\section*{Scaffold requirements}

Numerous scaffolds produced from a variety of biomaterials and manufactured using a plethora of fabrication techniques have been used in the field in attempts to regenerate different tissues and organs in the body. Regardless of the tissue type, a number of key considerations are important when designing or determining the suitability of a scaffold for use in tissue engineering:

(i) Biocompatibility

The very first criterion of any scaffold for tissue engineering is that it must be biocompatible; cells must adhere, function normally, and migrate onto the surface and eventually through the scaffold and begin to proliferate before laying down new matrix. After implantation, the scaffold or tissue engineered construct must elicit a negligible immune reaction in order to prevent it causing such a severe inflammatory response that it might reduce healing or cause rejection by the body.

(ii) Biodegradability

The objective of tissue engineering is to allow the body’s own cells, over time, to eventually replace the implanted scaffold or tissue engineered construct. Scaffolds and constructs, are not intended as permanent implants. The scaffold must therefore be biodegradable so as to allow cells to produce their own extracellular matrix\textsuperscript{5}. The by-products of this degradation should also be non-toxic and able to exit the body without interference with other organs. In order to allow degradation to occur in tandem with tissue formation, an inflammatory response combined with controlled infusion of cells such as macrophages is required. Now that tissue engineering strategies are entering clinical practice more routinely, the field of immunology is a playing a role of increasing prominence in the research area\textsuperscript{6,7}.

(iii) Mechanical properties

Ideally, the scaffold should have mechanical properties consistent with the anatomical site into which it is to be implanted and, from a practical perspective, it must be strong enough to allow surgical handling during implantation. While this is important in all tissues, it provides some challenges for cardiovascular and orthopedic applications specifically. Producing scaffolds with adequate mechanical properties is one of the great challenges in attempting to engineer bone or cartilage. For these tissues, the implanted scaffold must have sufficient mechanical integrity to function from the time of implantation to the completion of the remodeling process\textsuperscript{8}. A further challenge is that healing rates vary with age; for example, in young individuals, fractures normally heal to the point of weight-bearing in about six weeks, with complete mechanical integrity not returning until approximately one year after fracture, but in the elderly the rate of repair slows down. This too must be taken into account when designing scaffolds for orthopedic applications. However, as the field has evolved, it could be argued that too much focus has been placed on trying to develop scaffolds with mechanical properties similar to bone and cartilage. Many materials have been produced with good mechanical properties but to the detriment of retaining a high porosity and many materials, which have demonstrated potential \textit{in vitro} have failed when implanted \textit{in vivo} due to
insufficient capacity for vascularization. It is clear that a balance between mechanical properties and porous architecture sufficient to allow cell infiltration and vascularization is key to the success of any scaffold.

(iv) Scaffold architecture
The architecture of scaffolds used for tissue engineering is of critical importance. Scaffolds should have an interconnected pore structure and high porosity to ensure cellular penetration and adequate diffusion of nutrients to cells within the construct and to the extra-cellular matrix formed by these cells. Furthermore, a porous interconnected structure is required to allow diffusion of waste products out of the scaffold, and the products of scaffold degradation should be able to exit the body without interference with other organs and surrounding tissues. The issue of core degradation, arising from lack of vascularization and waste removal from the centre of tissue engineered constructs, is of major concern in the field of tissue engineering\(^9,10\). Another key component is the mean pore size of the scaffold. Cells primarily interact with scaffolds via chemical groups (ligands) on the material surface. Scaffolds synthesized from natural extracellular materials (e.g. collagen) naturally possess these ligands in the form of Arg-Gly-Asp (RGD) binding sequences (Fig. 2), whereas scaffolds made from synthetic materials may require deliberate incorporation of these ligands through, for example, protein adsorption. The ligand density is influenced by the specific surface area, i.e. the available surface within a pore to which cells can adhere. This depends on the mean pore size in the scaffold. The pores thus need to be large enough to allow cells to migrate into the structure, where they eventually become bound to the ligands within the scaffold, but small enough to establish a sufficiently high specific surface, leading to a minimal ligand density to allow efficient binding of a critical number of cells to the scaffold\(^11,12\). Therefore, for any scaffold, a critical range of pore sizes exists\(^13,14\) which may vary depending on the cell type used and tissue being engineered.

(v) Manufacturing technology
In order for a particular scaffold or tissue engineered construct to become clinically and commercially viable, it should be cost effective and it should be possible to scale-up from making one at a time in a research laboratory to small batch production\(^15\). The development of scalable manufacturing processes to good manufacturing practice (GMP) standard is critically important in ensuring successful translation of tissue engineering strategies to the clinic\(^16\). Another key factor is determining how a product will be delivered and made available to the clinician. This will determine how either the scaffold or the tissue engineered construct will be stored. Clinicians typically prefer off-the shelf availability without the requirement for extra surgical procedures in order to harvest cells prior to a number of weeks of \textit{in vitro} culture before implantation. However, for some tissue types, this is not possible and \textit{in vitro} engineering prior to implantation is required.

The final criterion for scaffolds in tissue engineering, and the one which all of the criteria listed above are dependent upon, is the choice of biomaterial from which the scaffold should be fabricated.

**Biomaterials**
In the first Consensus Conference of the European Society for Biomaterials (ESB) in 1976, a biomaterial was defined as ‘a nonviable material used in a medical device, intended to interact with biological systems’; however, the ESB’s current definition is a ‘material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body’. This subtle change in definition is indicative of how the field of biomaterials has evolved. Biomaterials have moved from merely interacting with the body to influencing biological processes toward the goal of tissue regeneration.
Typically, three individual groups of biomaterials, ceramics, synthetic polymers and natural polymers, are used in the fabrication of scaffolds for tissue engineering. Each of these individual biomaterial groups has specific advantages and, needless to say, disadvantages so the use of composite scaffolds comprised of different phases is becoming increasingly common. Although not generally used for soft tissue regeneration, there has been widespread use of ceramic scaffolds, such as hydroxyapatite (HA) and tri-calcium phosphate (TCP), for bone regeneration applications. Ceramic scaffolds are typically characterized by high mechanical stiffness (Young’s modulus), very low elasticity, and a hard brittle surface. From a bone perspective, they exhibit excellent biocompatibility due to their chemical and structural similarity to the mineral phase of native bone. The interactions of osteogenic cells with ceramics are important for bone regeneration as ceramics are known to enhance osteoblast differentiation and proliferation\textsuperscript{17,18}. Various ceramics have been used in dental and orthopedic surgery to fill bone defects and to coat metallic implant surfaces to improve implant integration with the host bone. However, their clinical applications for tissue engineering has been limited because of their brittleness, difficulty of shaping for implantation and new bone formed in a porous HA network cannot sustain the mechanical loading needed for remodeling\textsuperscript{19}. In addition, although HA is a primary constituent of bone and might seem ideal as a bone graft substitute, problems also exist in that it is difficult to control its degradation rate\textsuperscript{20,21}.

Numerous synthetic polymers have been used in the attempt to produce scaffolds including polystyrene, poly-l-lactic acid (PLLA), polyglycolic acid (PGA) and poly-dl-lactic-co-glycolic acid (PLGA). While these materials have shown much success as they can be fabricated with a tailored architecture, and their degradation characteristics controlled by varying the polymer itself or the composition of the individual polymer\textsuperscript{22-24}, they have drawbacks including the risk of rejection due to reduced bioactivity. In addition, concerns exist about the degradation process of PLLA and PGA as they degrade by hydrolysis, producing carbon dioxide and therefore lowering the local pH which can result in cell and tissue necrosis\textsuperscript{25}. The third commonly used approach is the use of biological materials as scaffold biomaterials. Biological materials such as collagen, various proteoglycans, alginate-based substrates and chitosan have all been used in the production of scaffolds for tissue engineering. Unlike synthetic polymer-based scaffolds, natural polymers are biologically active and typically promote excellent cell adhesion and growth. Furthermore, they are also biodegradable and so allow host cells, over time, to produce their own extracellular matrix and replace the degraded scaffold. However, fabricating scaffolds from biological materials with homogeneous and reproducible structures presents a challenge. In addition, the scaffolds generally have poor mechanical properties, which limits their use in, for example, load-bearing orthopedic applications.

The problems described above that exist with using scaffolds fabricated from a single phase biomaterial have resulted in much research being devoted to the development of composite scaffolds comprising a number of phases. For example, a number of groups have attempted to introduce ceramics into polymer-based scaffolds\textsuperscript{26-29} while others have combined synthetic polymers with natural polymers\textsuperscript{29,30} in order to enhance their biological capacity. While composite scaffolds such as these have shown some promise, each consists of at least one phase which is not found naturally in the body and they all have associated problems with biocompatibility, biodegradability or both. A more typical approach is the use of collagen-based scaffolds, either alone or with an additional phase incorporated to enhance biological and/or mechanical properties (Fig. 3). This is the approach used in our laboratory particularly
for bone regeneration but increasingly for other applications such as cartilage and cardiovascular repair.

**Case study: collagen scaffolds for bone tissue engineering**

Collagen is the most common protein in the body and provides strength and structural stability to tissues in the body including skin, blood vessels, tendon, cartilage and bone. Along with hydroxyapatite, collagen is one of the two major components of bone. It makes up 89% of the organic matrix and 32% of the volumetric composition of bone. As such, it has significant potential for culturing cells to produce bone. In our laboratory, we typically combine collagen (Type I) with glycosaminoglycan, a polysaccharide found in many tissues in the body, and, using a controlled freeze drying process, produce a highly porous collagen-GAG (CG) scaffold. The scaffolds we have developed are variants of the very first scaffold developed for tissue engineering applications. These were developed by Prof. Ioannis Yannas in MIT and received FDA approval to regenerate skin in burns patients. The development of the scaffold by Yannas, and subsequent clinical approval, was one of the key steps in the embryogenesis of the field of tissue engineering and led to the formation of Integra Life Sciences who still sell the CG dermal graft and are now one of the leading companies in regenerative medicine worldwide.

In common with all natural polymers, one major problem with using collagen as the main constituent of a scaffold for orthopedic tissue engineering is that it has relatively poor mechanical properties. However, we have demonstrated that the compressive and tensile mechanical properties of collagen and CG scaffolds can be improved through physical and chemical crosslinking methods. We have also identified the optimal composition for osteogenesis and optimal pore structure to facilitate bone tissue formation. Taken together, these studies have led to the development of a CG scaffold with an optimized composition, crosslinking density and pore size for bone regeneration and we have demonstrated the ability of these scaffolds to heal bone defects in vivo in minimally loaded calvarial defects. However, while these CG scaffolds have shown immense promise for bone repair in minimally weight-bearing regions of the body; in order to facilitate repair of regions where the scaffolds are subjected to higher levels of loading, we have strengthened these collagen-based scaffolds by introducing a ceramic phase and have thus developed a series of highly porous biomimetic collagen-hydroxyapatite (CHA) scaffolds (Fig. 3) based on the two primary constituents of bone.

These scaffolds not only possess significantly increased mechanical properties compared to a CG scaffold while retaining the highly porous and interconnected pore structure, but also show improved permeability which benefits cell infiltration and subsequent vascularization (Fig. 4). A comparative in vivo analysis between the CHA and CG scaffolds has demonstrated enhanced healing in the former scaffold. The reasons for this are two-fold: (i) the enhanced mechanical properties and permeability provided by the CHA scaffold allowed improved cellular infiltration and vascularization and (ii) the presence of calcium phosphate produced an osteoinductive response whereby its chemical composition enhanced the osteogenic potential of the host cells resulting in increased bone formation. The osteoinductive potential of calcium phosphates has been noted previously. The presence of the HA phase in these biomimetic scaffolds thus also imparts a bioinstructive facet to the materials. We also compared healing in cell-free scaffolds and scaffolds which had been cultured with mesenchymal stem cells (MSCs) for 4 weeks prior to implantation, i.e. an in vitro tissue engineered approach. Interestingly, we found reduced healing in the tissue engineered constructs which was caused, as revealed by immunological analysis, by excess matrix deposited by MSCs during in vitro culture, which adversely affected healing by acting
as a barrier to macrophage-led remodeling when implanted in vivo (Fig. 6). In other words, the study demonstrated that in addition to tissue engineered constructs failing after implantation because the scaffolds are insufficiently porous, they may also fail if the in vitro engineered tissue which has been formed on the scaffolds has been over-engineered leading to core degradation following implantation. This is consistent with a number of studies which have demonstrated how a major barrier to clinical success in the field of tissue engineering is this issue of core degradation\textsuperscript{9,10,50-52}.

**Scaffolds for tissue engineering: state of the art and future directions**

The challenge of tissue engineering is to mimic what happens in nature. Attempts are being made to engineer in vitro practically every tissue and organ in the body. Work is proceeding in creating tissue-engineered liver, nerve, kidney, intestine, pancreas and even heart muscle and valves. In the area of connective tissues, work has been ongoing worldwide for many years in the engineering of tendon, ligament, bone and cartilage. To date the highest rates of success have been achieved in the areas of skin\textsuperscript{11}, bladder\textsuperscript{53}, airway\textsuperscript{54} and bone\textsuperscript{55,56}, where tissue-engineered constructs have been used successfully in patients. In addition, autologous chondrocyte implantation (ACI) and matrix-induced autologous chondrocyte implantation (MACI) are showing some success for cartilage repair. This latter approach involves culturing harvested autologous chondrocytes on a collagen-based membrane for a number of weeks in Genzyme’s GMP-certified laboratories prior to the construct being shipped back to the patient’s hospital, followed by implantation at the injured site. This product is currently approved for use in Europe and Australasia.

While major breakthroughs have taken place and economic activity within the tissue engineering sector has grown exponentially, with increasing numbers of products entering the market place and into clinical trials, and with sales of regenerative biomaterials already exceeding US$240 million per annum\textsuperscript{57}, significant research is required in a number of specific areas in the field\textsuperscript{58}. As described above, lack of vascularity in scaffolds and tissue engineered constructs is a major challenge, and improving vascularization strategies is considered one of the areas requiring the most extensive research in the field of tissue engineering\textsuperscript{59-62}. One way to improve vascularization might be to engineer microvasculature by cells in the scaffolds prior to implantation and a large body of work in this area is ongoing by a number of groups\textsuperscript{62-65} including ourselves\textsuperscript{66} (Fig. 7). This is a complex approach and from a clinical perspective would involve initially harvesting cells from a patient/donor, then engineering a nascent microvasculature followed by engineering the desired matrix/tissue around this microvasculature; all of which would have to be achieved prior to implantation into a patient. Many clinicians question the efficacy of in vitro tissue engineering due to the requirement for at least two procedures and the delay in treatment while the construct is being cultured in vitro. From a commercial perspective, this approach also poses problems due to the prolonged regulatory process required before such a tissue engineered construct can be approved for clinical use. However, in tissues such as cartilage, which do not have the ability to regenerate themselves when damaged, long term in vitro tissue engineering may be the only solution to prevent the requirement of an eventual joint arthroplasty.

Other tissues, such as bone for example, have an intrinsic ability to repair, remodel and regenerate. The task in the field of tissue engineering is therefore to try and harness this innate regenerative capacity. One way to do so might be to engineer the scaffold in such a way that the scaffold itself provides regenerative signals to the cells which might negate the requirement for prolonged in vitro culture prior to implantation. Therefore, much ongoing research is devoted to developing more sophisticated biomimetic biomaterials with added
levels of complexity to incorporate multi-functionality and to encourage the biomaterials to have, for example, bioinstructive and stimuli-responsive properties (see review\textsuperscript{67}). Cells derive a vast wealth of information from their environment. The native extra-cellular matrix (ECM) surrounded, and produced, by cells is instructive, providing a dynamic and spatially heterogeneous constellation of microstructural, compositional and mechanical cues that can influence cell behavior. Harnessing the mechanosensitive capacity of cells, in particular, provides immense opportunities. The mechanical properties of tissues, biomaterials, cells, and biomolecules have profound biological consequences in terms of implant bioactivity versus failure, transmission of mechanical stimuli, and for a wide range of processes at the tissue, cell and subcellular levels. Key roles in molecular signaling pathways are played by cell adhesion complexes and the cellular cytoskeleton, whose contractile forces are transmitted through transcellular structures. Therefore, the mechanical properties of the substrate to which the cells are attached are critical to the regulation of cellular mechanotransduction and subsequent cellular behavior. This has important implications for development, differentiation, disease, and regeneration. It is now clear, for example, that substrate stiffness can regulate both the behavior of mature cells\textsuperscript{68,69} and the differentiation pathway of stem cells (see review\textsuperscript{70}). For example, when MSCs were grown on firm gels that mimic the elasticity of muscle, differentiation down a myogenic (muscle-forming) lineage was observed, whereas when MSCs were grown on rigid gels that mimic pre-calcified bone the cells differentiated down an osteogenic pathway\textsuperscript{71}. Similarly, with neural stem cells, neuron differentiation is favored on soft scaffolds that mimic normal brain tissue, whereas differentiation into neuron-supporting glial cells is promoted on harder matrices\textsuperscript{72}. In addition, cardiomyocytes (heart muscle cells) have been shown to beat best on a substrate with heart-like elasticity, whereas on harder substrates, that mechanically mimic a post-infarct fibrotic scar, cells overstrain themselves and stop beating\textsuperscript{73}. Therefore, increasing research is now being directed at utilizing the mechanosensitive capacity of cells to develop scaffolds and biomaterials with specific mechanical properties which can be used to direct the behavior of the cells with which they interact\textsuperscript{36,74-77}.

In addition to biomechanical signals, cellular behavior is strongly influenced by biological and biochemical signals from the extracellular matrix. Therefore, the use of scaffolds as delivery systems for growth factors, adhesion peptides and cytokines is receiving considerable attention in the field\textsuperscript{67,70,78,79}. The incorporation of, for example, angiogenic growth factors in scaffolds might also improve their vascular potential. Similarly, methods of providing nerve supply to newly formed tissues also need to be considered\textsuperscript{80}. Another area of critical importance is controlling, and understanding, the host immune response and preventing infection following implantation. To this end, the incorporation of drugs (i.e. inflammatory inhibitors and/or antibiotics) into scaffolds has been proposed as a method to reduce the possibility of infection after surgery\textsuperscript{81}. Finally, the use of scaffolds as delivery systems for therapeutic genes is undergoing considerable investigation\textsuperscript{82-87}. Gene therapy approaches (viral and non-viral) which utilize DNA encoding for therapeutic genes potentially provide a more stable and effective approach to allow sustained and controlled release of therapeutic factors. Gene therapy can thus be a valuable tool to avoid the limitations of the local delivery of growth factors, including the short half-life, large dose requirement, high cost, need for repeated applications, and poor distribution\textsuperscript{88}. The idea of a gene delivery vector contained within a scaffold, although not new, is a recent development in the field of regenerative medicine and the system has been coined as a ‘gene activated matrix’ (GAM) by Bonadio and co-authors\textsuperscript{89} who developed the very first system. Despite the prolonged regulatory process required to enter the clinical arena, such a GAM might offer
advantages by increasing the rate of DNA delivery into the cells which are in contact with the
gene-eluting scaffold and provide spatial and temporal control of the desired gene.

These areas of new and expanding research demonstrate just how multidisciplinary the field
of tissue engineering has become and while the challenges are vast, the opportunities for
improving human health in a whole variety of areas are immense. Undoubtedly exciting times
lie ahead in this field, which is only now beginning to define itself as more technologies enter
the clinical and commercial arenas.

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Figure Captions

Fig. 1. Tissue engineering triad of cells, signals (provided chemically by growth factors or physically by a bioreactor), and the scaffold which acts as a template for tissue formation by allowing cells to migrate, adhere, and produce tissue.

Fig. 2. Confocal micrograph showing osteoblast cells (green) attached to a highly porous collagen-GAG scaffold (red). The mechanism by which cells attach to biomaterials and scaffolds for tissue engineering is critically important for successful tissue regeneration.

Fig. 3. Comparative SEM images of (a) collagen-GAG (CG) scaffold (b) hydroxyapatite (HA) and (c) composite collagen-HA (CHA) scaffold. The high porosity of the CG scaffold, which promotes improved cell infiltration and vascularization, is evident. The drawback is that it has poor mechanical properties. The HA scaffold has better mechanical properties but poorer capacity for cell infiltration and vascularization. By producing a CHA scaffold (c), it is possible to overcome the problems with both materials while retaining their positive attributes. The high porosity of the CHA scaffold and uniform distribution of HA particles (green dots) can clearly be seen. (a) Reproduced with permission from 90, (b) Reproduced with permission from 52.

Fig. 4. Effect of hydroxyapatite addition on (a) stiffness and (b) permeability of collagen scaffolds. The addition of hydroxyapatite results in a significant increase in stiffness (*p < 0.05) but also helps to improve the permeability, as the scaffold pores tend to remain open with no collapse following hydration. This promotes improved cell infiltration and subsequent vascularization after implantation. Reproduced from 45.

Fig. 5. Quantitative cell-mediated mineralization by osteoblasts on the CHA scaffolds containing differing amounts of HA (expressed as % weight with respect to collagen). ‘Blank’ shows original amounts of HA in the scaffolds for comparison. The presence of HA produced an osteoinductive response whereby its chemical composition enhanced the osteogenic potential of the host cells resulting in increased bone formation. Reproduced from 45.

Fig. 6. Example of core degradation in a rat calvarial defect treated with a tissue engineered collagen-calcium phosphate scaffold 4 weeks post implantation. Fig. 6(a) shows the full defect area. It can be seen that there is significant inflammation and a capsule (red arrows) has formed around the periphery of the implanted tissue engineered construct resulting in core degradation. Fig. 6(a) shows a higher magnification image of the defect area and it can be seen that the core region is completely acellular (black arrows). White arrows represent original host bone. Reproduced with permission from 7.

Fig. 7. In vitro microvessel formation by endothelial cells on the CG scaffold. In this image, cell-seeded constructs were labeled with AlexaFluor 488 Phalloidin (which stains the cell cytoskeleton green) and DAPI (which stains the cell nucleus purple). Vessel formation was then observed using multi photon imaging.
**Instrument Citations**

Fig. 2: Zeiss LSM-510 confocal microscope
Fig. 3: JEOL JSM-5410 LV scanning electron microscope
Fig. 4: Zwick/Roell Z050 mechanical testing machine and permeability measurements were carried out using an in-house developed testing rig\(^3\)
Fig. 5: Titerek Multiskan MCC/340 spectrometer
Fig. 6: Nikon Eclipse 90i epifluorescence microscope
Fig. 7: Zeiss LSM 710 multi-photon imaging system.
Fig. 1
Fig. 4
Cell-mediated Mineralisation

Fig. 5