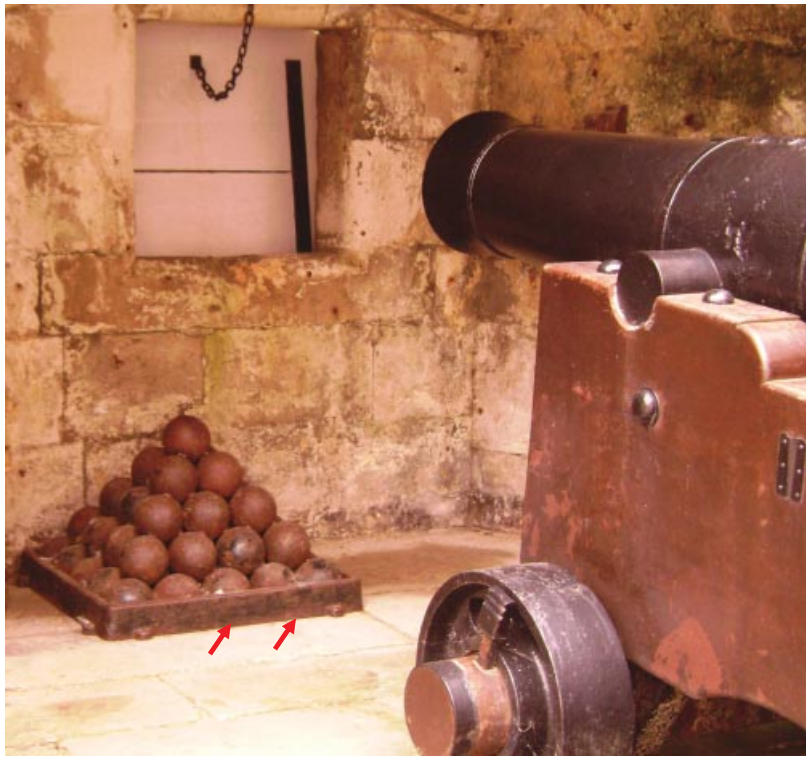


Understanding the scale where we are operating is absolutely critical when we consider 3D shape and spatial organisation. This pyramid of cannonballs forms a splendid 3D shape example. Held together by the square brass 'monkey' (arrowed), it has generated a legend and common English saying. The trouble is that when we actually *measure* at the scale that matters, the legend seems not to stack up.

5 Making the Shapes for Cells in Support-Scaffolds

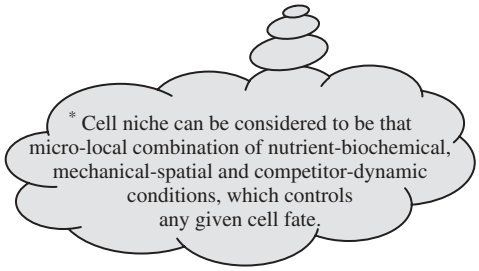
Constructing tiny Galapagos for cells

In basing so many of his illustrations of the evolution of niches on the very special Galapagos Islands, Darwin made a particularly intelligent selection. The island location makes the local niches excitingly special, capturing and engaging the imagination. At the same time, the special effect of being an isolated island group has tended to greatly simplify the picture we see. In other, contiguous locations in a large land mass, the same process, repeated over the millennia, has left an unthinkable complexity which we still pick over.

Multi-dimensional complexity is the essence of niche evolution in bio-systems, but looking full-face into that complexity is too daunting to understand. Like studying the sun during an eclipse, Darwin's genius was to illustrate the evolution of niche complexity through simplified versions – on remote ancient islands.

Extreme Tissue Engineering: Concepts and Strategies for Tissue Fabrication, First Edition. Robert A. Brown.

In our small way, extreme tissue engineers are trying to understand and, for short periods to recreate, *grossly simplified* cell niches* in order to achieve



our bio-fabrication goals. We are trying to build small, isolated but biomimetic 'islands of cell-space' with our cells in situ and working for us. In other words, we are aiming to build our equivalent of a Galapagos – simple enough for us to cope with, given that the full niche is so mind-meltingly complex. Here, we take a look at ways in which we can and are making Galapagos at the cell-scale.

5.1 3D shape and the size hierarchy of support materials

There is a saying in English when the weather gets really cold that it 'could freeze the balls on (or off) a brass monkey' – commonly shortened to '*brass monkey weather*'. Legend has it that this saying comes from the old naval days where cannon balls were stacked in pyramid shapes by the side of their guns. Triangular trays with holes machined to be close-fitting and grip the cannon balls would hold each layer and so keep the pyramid of balls together (for an illustration, see any good Hollywood pirate film). These were known as 'monkeys' and made from brass (front piece illustration). The story was that at very low temperatures, the difference in thermal contraction between the steel balls and brass monkey would either force off the balls or make them stick (in both cases rendering the warship useless).

This is a rather quaint analogy for our cells in their neatly fitting niches within an engineered 3D structure. Providing the physical shape and dimensions of the supporting material are made appropriate to the cells or cannonballs, they will be sustained in a stable, 'comfort' position and will thus be ready to perform their function when needed. The importance of 3D space and shape is equally highlighted in this analogy by the *apparently* subtle temperature-sensitivity of the (brass monkey) support-system.

The trouble here is that this story is now regarded as an urban myth (or Hollywood pirate-story), for many reasons. First, in reality cannonballs were stored safely below decks in long *wooden* trays ('shot garlands' – i.e. no metal-to-metal-anomalies). Also, a fatal flaw in the story is that the difference in brass-iron expansion coefficients is small, producing only $\approx$1 mm change over a 1 metre long monkey if the temperature changed by 100°C!

The moral of this is that if we want to produce control systems with shape and 3D structure, it is essential to do the measurements and find out *exactly* which part of the length-scale hierarchy our cells (or cannonballs) are sensitive to. Hollywood can prosper from arm-waving ideas, but tissue engineers will not.

Figure 5.1 shows a scanning electron micrograph of a collagen gel (very biomimetic) embossed with a pattern, derived from a fine fibre mesh. At the gross, millimetre-scale (top), the 3D pattern resembles that of the original embossing mesh. It is an orthogonal pattern, with opposing fibre strands arranged at right angles. As we look at higher magnification, in the in 1–100 µm scale (middle panel), the pattern becomes strongly parallel. In the lower panel it is clear that, at the sub-µm scale, the 30–50 nm diameter collagen fibrils which make up each ridge are completely random in orientation. Not visible here (but drawn), there is a further level where collagen molecules which make up the fibrils (1–2 nm diameter) again have a parallel alignment for significant distances, as this is how the molecules aggregate into fibrils.

In other words, the **same structure** has at least four distinct patterns of 3D orientation, depending on the scale-hierarchy that is sampled. There is, then, a '**reading-window**' effect for any given bio-structural hierarchy, which makes it important to consider at which scale these structures are being read (i.e. the scale at which they act). A single cell would mainly sample and utilize structures

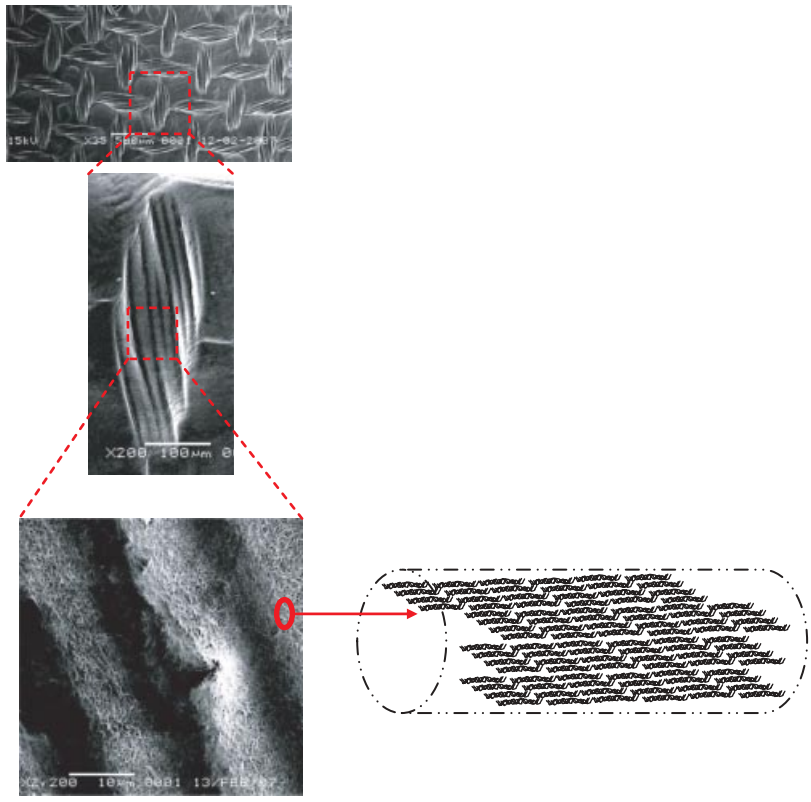


Figure 5.1 A pattern embossed into a single, dense-compressed native collagen gel. Scanning electron micrographs of the same site (embossed with nylon mesh), but at three increasing enlargements. Top, bar = 500 µm; middle, bar = 100 µm; bottom, bar = 10 µm). The diagram (bottom right) shows the 'parallel' pattern of collagen molecular packing within each of the fibrils (i.e. the fine strands just visible in bottom micrograph). From: Kureshi, A., Cheema, U., Alekseeva, T., Cambrey, A. & Brown, R. (2010). Alignment hierarchies: engineering architecture from the nanometre to the micrometre scale. *Journal of the Royal Society Interface* **7**, S707–S716.

in the middle range of this hierarchy. In contrast, the integrin receptors embedded within the cell membrane would bind to 3D structures in the mid-nm range (lower panel). Only where cells form larger clusters, or syncytia, could they utilize mm-scale patterns.

Finally, at the other end of the spectrum, parallel macro-molecular structures in the low nanometre range of the hierarchy might influence protein-protein interactions and glucose mass transport. The hierarchy paradox means that, like expansion and cannonballs, 3D structure does not matter simply because we can see it. This is a function of our microscopy. 3D structure of support materials *must* be measurable to the cells that we hope will use it. If the scale of the structural cues is too large or too small, they cannot affect cells directly. Like the sound of a dog-whistle to the human ear, it might as well not exist.

The take-home message here, like so many before, is pretty obvious once stated, but no less important to actually implement. Our task here is (simplified) 3D niche design for cells but, to achieve this successfully, it is critical to get our minds down to the cell and molecular level in the scale hierarchy. Not least it is necessary to measure features and events we have engineered around our cells in order to make sure that our designs really do work as we expect. This is, after all, just good hypothesis testing. The surprise perhaps, is that we still have so much basic biology to learn after so many centuries of study.

Then again, that is what followed from Darwin's beautiful simplification.

5.2 What do we *think* 'substrate shape' might control?

Cell-support architecture is basically the way that the bulk composition is structured in 3D to hang together in space. This also governs how the surfaces are made available for our cells to hold onto and grow over, either alone or attached to their neighbours. That same 3D organisation and pattern of surfaces also dictates the rate and direction that soluble molecules (nutrients, oxygen, proteins) move, enter and leave. If that were not enough, support-material architecture also controls mechanical properties of our constructs, particularly at the cell/micro-scale.

To use a human scale analogy, it is easy to understand why the material properties of glass lead to its use in particular functions. Think of glass and we think 'hard, brittle, optical clarity'. But it is still striking to look at the wide variety of detailed functional properties which can be achieved by fabricating different μ-structures. Figure 5.2 suggests some of these, from the transparent table top to opaque etched decoration and the sharp microparticle abrasive paper.

Clearly, then, chemical composition matters, particularly in the basic and the gross functions, but 3D spatial organisation of that material across the length-hierarchies dominates the more subtle and dynamic properties. So, if we agree that the extreme challenge is to reproduce *some* of the subtlety and dynamics of native biology, where would you, the reader, choose to concentrate your efforts?

To list and organize the 'tissue-engineering-critical' properties which are controlled by architecture, we can start at the sub-nano/molecular level and float up the size hierarchies. At this level, small nutrient/metabolite molecules such as sugars, amino acids, phosphates and O_2/CO_2 pass rather easily through different nano-structures. However, the direction and rate of movement is dictated by the packing and alignment of the bulk material.

Figure 5.2 The same composition of glass is shown here, used in a number of different 3D architectures. This makes a reflective glass mirror and transparent table surface (i) smooth, down to the nm scale; (ii) opaque, with μm-scale etched surfaces (decoration on the decanter) and (iii) abrasive glass paper comprising mm-scale random glass fragment particles, for scouring painted surfaces (i.e. the 'green' glass-paper, rear). Although their compositions are the same, they each have contradictory properties and completely different functions, based on the scale of their surface structure (ultra-smooth versus abrasive; transparent versus opaque).

The effects of bulk structure on small molecule movement at this level can also be dramatically affected by distortions of the structure during dynamic mechanical loading (Figure 5.3).

The same factors affect mass transport of control macromolecules, such as proteins, though at a different scale – in the nano/sub-micron range. Such protein movements dominate export, import, remodelling processes and repair. This, in turn, means that support-material structure controls cell function (cell-matrix attachment, cytoskeleton, shape, division, differentiation) plus communication and cooperative behaviours. In turn it will influence cell-cell attachment & dialogue, migration, sheet/layer formation, contraction, fusion, synapsis).

So, aside from that little lot, how else *could* 3D μ-structure affect the things we are most concerned about in tissue engineering?

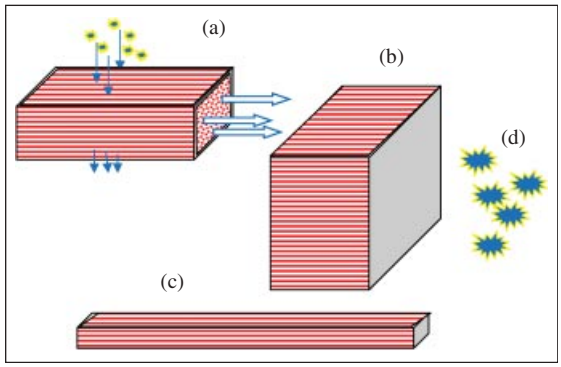


Figure 5.3 Where small molecules are passing through a fine, aligned fibre structure they may pass freely across the structure or be deflected. In (a), small molecules entering from the upper surface are shown being 'deflected' orthogonally, moving more parallel to than between the fibres (i.e. path of least resistance where size shape is a constraint). Smaller molecules might be expected to diffuse more equally in all directions. What, then, would you expect to happen to molecular movement when the same material is deformed (compressed) to be shorter and fatter (b) or stretched to be longer and thinner (c) – or where it was cycled, slowing from one extreme to the other? Alternatively, what would happen if the diffusing molecules had an average radius 10 or 50 fold greater (d)?

5.3 How we fabricate tissue structures affects what we get out in the end: bottom up or top down?

How we fabricate tissues is the subject of later chapters, but at this stage it is worth outlining, at the most general level, which overall strategies are available to us. This highest level of strategy refers to the '*direction*' in which the chosen fabrication route occurs: **top-down** or **bottom-up**.

Production of petroleum products from crude oil may be considered as *top-down* processing, whereby highly complex starting material is refined down and sometimes modified into its useful components. Starting materials vary from 'Brent crude' and 'West Texas Intermediate' to heavy crude, bitumen and tar. Depending on their viscosity, complexity and sulphur content, they can be used to produce different products. If, or when, it becomes economic to manufacture synthetic fuels by catalytic assembly of natural methane, or even from elemental carbon and hydrogen, this would be a *bottom-up* process.

In short, we can either *refine down* complex (often natural) products or *build up* from simple purified building blocks. Importantly, in both directions, there can be many starting points, depending on what we choose as the 'top' or the 'bottom'. The top can be unbelievably complex and messy, while the bottom can be uneconomically and atomically basic and pure, but at each extreme the costs and technical difficulties soar. It is not accidental that when petroleum was cheap, our ancestors did not bother refining the prolific tar-shale deposits that we now use.

The crude oil analogy here, however, is incomplete as it only covers chemical composition. We are considering here assembly of 3D materials with stable shape and substance. In the case of fabricating shape, we might consider making a boat by hollowing out tree trunks as a top-down approach. The bottom-up alternative would then be to make and shape a clinker-built boat with nails and smooth planks. Similarly, digging passages, doorways and rooms into soft rock is top-down house building, while manufacturing rectangular bricks, tiles and cement is how we generate a bottom-up housing market (Figure 5.4).

These analogies teach us that there is a utility-tension between these two approaches. Top-down tends to be simple and economic, leaning heavily on what is easily available, but using crude starting shapes and materials. The relative simplicity of this fabrication has to be balanced against the restrictions on what you can do (i.e. the starting material dictates what is possible). By contrast, bottom-up shifts the effort and expenditure to making and assembling the material building-block. This is initially more complex and expensive, but this investment opens up the possibility of making *exactly* what is needed, where it is needed.

Cell support materials which are made predominantly from synthetic polymers (e.g. poly-lactides or poly-glycolic acids) are, almost by definition, made by bottom-up processing. In this case, we go from simple chemical monomers assembled into

Figure 5.4 The building of **cave houses**, as opposed to free-standing brick or wood houses, can be viewed as two contrasting construction methods. The first (cave house building) represents a top-down form of construction, where an existing complex 3D structure (the rock cave) is cut out, selectively dug away and simplified down to give the desired shape and function. On the other hand, a free-standing house is fabricated bottom-up, from nothing, using basic building materials of bricks, planks and tiles. The difference is clear in these cave houses in Southern Spain, where (a) is a home built into a rocky outcrop; its chimney (**) and garden (b) show the idea. Larger dwellings are seen in larger rock faces (c), where the entrance (red arrow) and patio (open arrow) might look like a standard house, but the upper windows and balcony (double arrow) give it away.

polymer chains and then aggregated to fibres or sheets for assembly into 3D structures. Mineral materials, such as hydroxyapatite, can be made top-down by grinding up extracted bone mineral into powders, but they are more commonly built up from their inorganic chemical constituents.

Natural protein materials, however, present a far greater variety of options, illustrated here by extending the collagen example which we started in the last chapter under 'composition'. Possibly the best example of the two-directional, bottom up/top-down approach to engineering materials structure can be seen in collagen-based cell support materials. There are two counter-current logics. The first relies on **refinement down** of whole (collagen-rich) tissue structure, as against **assembly up** of simple collagen building blocks (Text Box 5.1).

The top-down approach uses native animal tissues, with all their advantages of natural architecture, strength etc. Top-down processing involves the removal of as much as possible of the animal cells, cell debris and other antigens, minimizing risks of rejection or infection. Although some versions are in clinical use, this remains 'work in progress' to improve clearance and prevent unwanted inflammatory responses. At its best, this tissue (top-) down approach generates excellent biomimetic structure with exceptionally strong and biocompatible properties, ready for cell seeding. This is exemplified by the processing of porcine small intestinal submucosa – SIS – to provide implantable platforms, some of which are currently in use clinically.

For top-down fabrication it is essential that the animal cells of the original tissue are broken down into small enough fragments to allow these to escape from the dense tissue mesh of matrix which is required. However, as Shakespeare might have put it, 'there's the rub![7]' Given that the natural pores, tracks and channels in these dense native connective tissues are mainly nano-scale to a few μm, there is little chance to force multi-micron scale

[7]'*To die to sleep, to sleep, perchance to dream; ay, there's the rub* . . . ' Part of the 'To be or not to be . . . .' soliloquy in Shakespeare's *Hamlet*. 'Rub' was a sporting term for an obstacle in the game of bowls which diverts a ball from its true course.

Text Box 5.1 Differences of opinion about what the 'bottom' is in bottom-up

'Bottom-up' and 'top-down' are terms more common to fabrication technologies and engineering than the biosciences (i.e. the biology-tribe members). Perhaps predictably, this means that the complex-top can be much more complex than we are accustomed to in engineering. For example, we probably need to recalibrate the concept of 'complex' versus 'simple' where biologists are involved. Their natural reaction is that, despite its complexity and variability, a cell can be considered as a 'basic building unit'. The justification here is that to qualify as 'living', we are pretty well stuck with cells, however imperfect, they are as *the* bottom unit.

This recalibration is just as necessary for our definition of what we choose to class as 'bottom' for protein materials. For example, in the case of collagen, it can be taken as reasonable that the native collagen monomer is the basic building block for the material. However, to go up a scale in 'basics', pre-aggregated collagen fibrils could be used as base units to build our materials with a new bottom-up. On the other hand, to go down a scale, we *could* assemble strings of collagen-like amino acid sequences into designer proteins with some of the properties of collagen materials.

The question is, since amino acid sequences are smaller units, are they then the true bottom, from which we go up? The answer is, 'not really'. These are crude distinctions based on process 'direction', and there is no moral high ground in having a more basic or smaller bottom – just the normal rewards of pragmatic function. In fact, if this were true, the peptide guys would be undercut by organic chemists synthesizing amino acids from carbon and nitrogen.

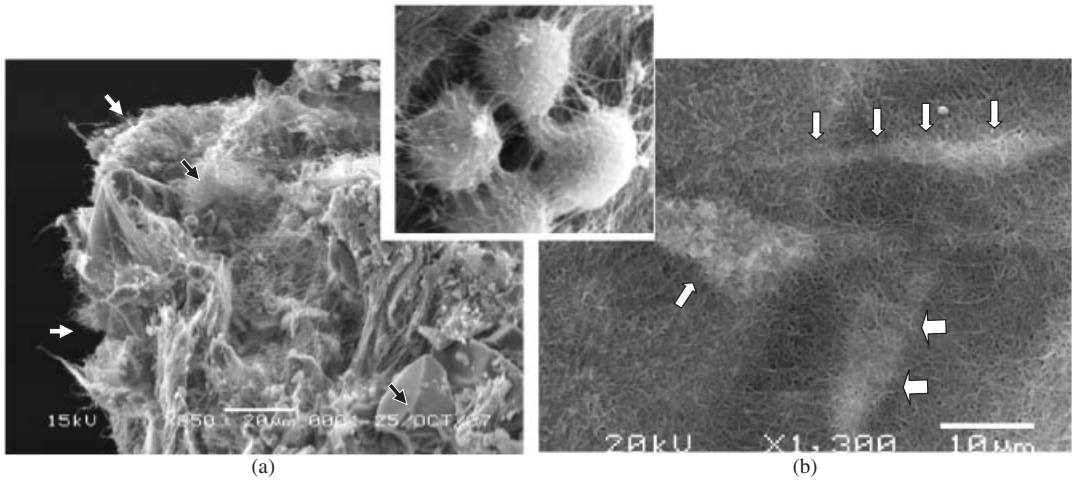
If any reasonably definable basic building blocks allow us to assemble what we need, economically and reproducibly, that is OK. In the example here, synthesizing complex collagen protein 3D materials from isolated amino acids is not practical, economic or functional at present. At the other extreme, large fibril aggregates, in the form of shredded collagen tissue fragments, are certainly cost-effective and simple, but they can be functionally limited. This presently favours the use of intermediate level starting units, namely collagen protein monomers aggregated into fibrils around cells. However, in reality, the base point changes with time as it is sensitive to technology and society's expectations.

cells into that mesh when it comes to re-seeding without substantive disruption. The trouble is that 'substantive destruction' of the native architecture reduces the great advantage of the top-down processing. Current approaches to achieve this interstitial seeding (into the collagen mesh) is either to wait until incoming cells break open their own multitude of pathways into the bulk collagen (often *in vivo*), or to cause substantial artificial disruption to the mesh using detergents or ultra-sound.

In contrast, using the bottom-up route, collagen is assembled from small, monomer or oligomer units (Text Box 5.1). The collagen nano-fibrils aggregated in this way are far smaller than the cells that they enmesh, just as they are in living tissues (Figure 5.5b). This is made easy where collagen fibril assembly, or gelling in general, occurs under physiological (i.e. cell-friendly) conditions, as happens around any resident cells. The result is that cells are seeded **interstitially** (i.e. into the fabric of the fibril material) from time zero.

In terms of our previous 'composition' distinction between synthetic biodegradable polymers versus cell-degradable natural polymers, there are clear consequences for the success of our fabrication of μ-structure. Not least, the dynamic of controlling the μ-structure of the natural polymers is immediately given over to the resident cells to develop, change and remodel, as they are programmed to do. In contrast, where resident cells have no ability to remove synthetic polymer structure, their ability to perform remodelling processes is restricted or even removed. This means that the bottom-up effect of being able to fabricate nano-structured support matrices, with cells enmeshed at t_0, is complemented (using protein supports) by enabling cells to maintain the matrix biomimetic structure as time progresses (t_{0+n}).

So, in short, synthetic, bio-soluble polymers block natural cell-tissue dynamics, whereas natural polymer cell-supports give up the dynamic of process-control to the cells. Note: the risk of



(a) (b)

Figure 5.5 (a) Soluble phosphate glass particles, <20 μm (black arrows), embedded in a nano-fibrillar collagen gel (white arrows), compressed to a tissue-like density. (b) Cells interstitially seeded into the fabric of the collagen fibril mesh-work, and again compressed to a tissue-like density. Main picture shows three enmeshed fibroblasts just below the surface (arrows); inset shows a group of four cells just retained by a few surface collagen fibrils. Reproduced by kind permission of Dr. Tijna Alekseeva, UCL.

giving up control is that cells can (and do) get the programme 'wrong', especially *ex vivo*.

5.4 What shall we seed into our cell-support materials?

We now come to a major tissue engineering-scaffold distinction which we have seen emerging more and more regularly in the previous sections. This is the way in which some materials can be assembled and formed into 3D shapes *around* resident cells, while others must be *seeded* with cells after fabrication. The former can be described as 'interstitial-' or even t_0 (time-zero) seeding, as opposed to the latter, 'surface-' or post-fabrication seeding.

Some readers may already be starting to see why the stage and manner of cell seeding is, in fact, one of the most important factors in determining the level and type of 3D structuring we can achieve. Once again, it comes down to the scale hierarchy question. Most mammalian cells we would like to use range in size from 12 to 25 μm in diameter (when spherical). Thus, for cells seeded post-fabrication, some form of access 'pores' of 100 μm or more will be needed to allow even small cell groups to penetrate to deeper zones. 10 μm apertures might allow the squeeze-through of single flattened cells and so painfully slow seeding. The result is that post-fabrication seeding materials have 3D μ-structures *forced on them* in a manner which has nothing to do with biomimetics, bio-control or cell function. In contrast, interstitially t_0-seeded materials are *not* forced to grow in this way.

The cargo-carrying ship analogy of the last chapter is one we should continue to develop. Interestingly, that cargo does not necessarily have to be cells alone. Depending on the nature of the cell support and its intended application, there are many ways of loading with nano-micron scale particles, macromolecules (hormones, growth factors and enzymes) or even small molecule cargos (e.g. drugs or metabolites). Fibrous cell-supports, formed by gelling under physiological conditions, permit the widest range of interstitially seeded cargo-carrying options. More robust cargos, such as hydroxyapatite particles, mimicking hard tissue substrates such as bone, can be added to delicate, natural protein materials as well as those made under cell-lethal temperature, pH or solvents.

Figure 5.5a shows the appearance of soluble glass particles (<20 μm diameter) cast into a collagen gel

after compression to near-tissue matrix density. Larger or heavily charged bio-molecules (typically >100 k molecular weight) can be incorporated directly into the fabric of many support materials – for example, those with a nano-fibrous mesh or pore structure with slow or cell-dependent breakdown. However, in many cases, delicate or smaller macromolecules, drugs and metabolites can be carried in artificial vesicles or nano-particles, which are themselves trapped within the support material. Such delicate cargos commonly need to be loaded under physiological conditions, either to preserve the cargo itself or its vesicle wrapping. Again, this can be simpler for gelling and nano-porous support structures.

The use of support materials which carry alternative bio-control cargos in addition to their complement of cells has grown in importance with the need for complex, agent-delivery systems at the implant site. This is increasingly seen as a major point of contact with conventional pharmacological and bio-molecular therapies, and a rich opportunity for collaboration with the 'pharmacology' tribes.

Continuing our extended example analysis of collagen-based cell-support materials, it is clear that cells can be seeded onto the surfaces of top-down fabricated, whole tissue-derived materials such as SIS (see Section 5.3). Bottom-up fabrication of cross-linked collagen sponges, involving freeze drying or glutaraldehyde treatments, must still be seeded post-fabrication. However, supports made using native fibril self-assembly (i.e. native collagen or other self-assembling hydro-gels) aggregate at the nano-scale, around cells, to give tissue-like 3D cell-matrix structure. This is interstitial cell distribution within the material in a nano-fibrous mesh.

5.4.1 Cell loading: guiding the willing, bribing the reluctant or trapping the unwary?

We are about to analyze shipping analogies involving human cargos. With the aim of side-stepping any political correctness sensitivities which might raise nasty historic or geo-political connotations of 'mass transport of people', we shall specify 'troop ship' or 'worker's ferry'.

Clearly, once we reach the point of loading living cells as part of our cargo, we are well inside the zone requiring delicate biological treatment conditions. This is much the same as for passenger transportation, where cabin space and leisure conditions are critical. Paradoxically, loading of cells *can* be easier than non-living cargos, because cells will, under the right conditions, move into the deeper parts, much as passengers will distribute between decks, given incentives, time and a map. Without good instructions, though, both cells and passengers are as likely to go anywhere they fancy – or nowhere.

Much of the earliest work in engineering tissues, using synthetic polymer cell-supports such as PLA and PLGA, was designed to encourage cells to migrate, as far and as fast as possible, from the surfaces where they were seeded, to the deeper zones. Once cells had attached to the surface, the aim was to make it as easy as possible for them to populate all levels of the construct. This affected how 'pores' were viewed and designed. The architecture of these cell-supports was fabricated with this as the primary aim, giving rise to porous foams, non-woven, woven and knitted meshes. Fibre-based materials tended to produce structures with complex, sometimes highly asymmetric patterns, comprising interconnected pores or channels with a huge range of sizes and shapes.

The variability and pattern of these depends on whether the fibres are woven, non-woven, knitted or completely random-enmeshed (like cotton wool) and on the variability of fibre diameter. Non-fibre-based materials can have more regular pore structures, described numerically as mean pore diameter, percentage porosity or interconnectivity. These terms are found commonly in descriptions of sponges or films used to support cells, where pore distribution can be:

(i) homogeneous throughout;
(ii) larger in superficial areas; or, in some instances,
(iii) laser-drilled from top to bottom through otherwise dense films (e.g. Hyaluronan: 'Laserskin').

For these materials, pore density, diameter and connectivity are important features, though notably, these range widely across the scale of several 100 μm, i.e. many cell diameters.

In terms of our troop-transport analogy, this would be a little like designing a vessel with huge open spaces, so that the troops can get on board as fast as possible (Figure 5.7). However, large, open living spaces are not so great for the occupants during long, rough sea crossings, unless it is assumed that the troops will reshape the space once they are aboard. In a way, this was the logic behind early cell-supports: get the cells to fill all the 3D holding spaces *as fast as possible* and assume that they will construct what they need to 'be comfortable' for the longer term. Unfortunately, this assumption *is wrong*. It might happen where the troops have ambiguous, vague instructions but no detailed operation manual (in the case of cells, read 'ambiguous control cues'). Alternatively, the problem might be that building the necessary 3D comfort structures, cell remodelling, takes the troops or cells too long and the mission goes 'wonky'. The troops are forced to roll about pointlessly in unsuitable spaces for weeks, arriving exhausted, sick or mutinous–just like our cells.

Clearly, there is a place for wide open spaces to transport close-packed troops, but this would be a 'landing craft' (Figure 5.7), which has a completely different philosophy and purpose. The name in this case is the clue: landing crafts are designed for short, quick, on-off *landings* (maximum duration, hours). Critically, where this is the *primary* design aim, there can be no assumption that the troops will do anything other than 'be transported'. So, just as with troop transportation, it is important to work out exactly what we want our cells to achieve *before* they are pushed aboard our cell-support constructs. This includes pre-launch understanding of exactly what we can *realistically* expect our cells to contribute to the process.

To wind up the cargo ship analogy, it is clear that to achieve simple, maximal *carrying capacity* (whether cells, troops, bananas, oil or gravel) the best designs will have the least internal 'dead space' created by walls, floors, corridors and partitions. In effect, the empty cargo space *can* be arranged, either as one large void or as thousands of tiny sections. But the overall ship volume will carry much more if the 'cargo' is in one or two huge compartments, as it is in the case of oil or gravel.

On the other hand, as we saw earlier, **how the cargo arrives** matters. Bananas do not travel so well in five metre high piles. People and cells are even less tolerant of rush-hour transport conditions for more than a few minutes. This, then, represents a very real tension. People or cell-cargos can travel long periods in single, well supplied and tailored compartments (Figure 5.6) or crushed together *en mass* for very short periods (Figure 5.7). Critically, this factor must take priority over simple carrying efficiency, otherwise nasty (surprising) things happen to the cargo. After all, both human and cellular cargoes have a 'choice'. So let us have a closer look at why this is the case.

The special thing about soft, living cargos (i.e. not gravel/oil) is that they *must* be supplied with

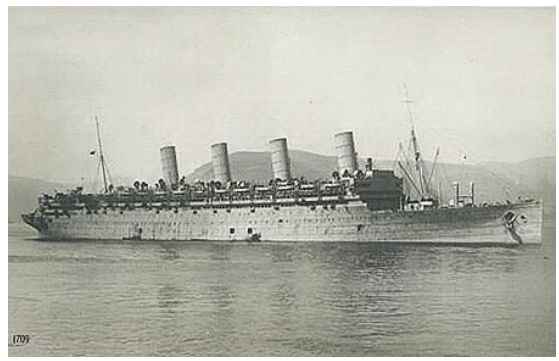


Figure 5.6 The Cunard liner *Aquitania* was used for long-distance troop transport in World War II. Men travelled in cabins designed for passengers as well as occupying cramped space between the crew and the engines. Provided the men were tightly controlled (i.e. obeyed orders), they kept to their windowless cabins deep in the ship and away from the decks. However, mutinous, overcrowded troops in hot weather will push themselves from the bowels of the ship onto the deck or to the best cabins, unless enticed with some creature comforts. And so it is with cells within the interior of a deep material. © Cunard Line.

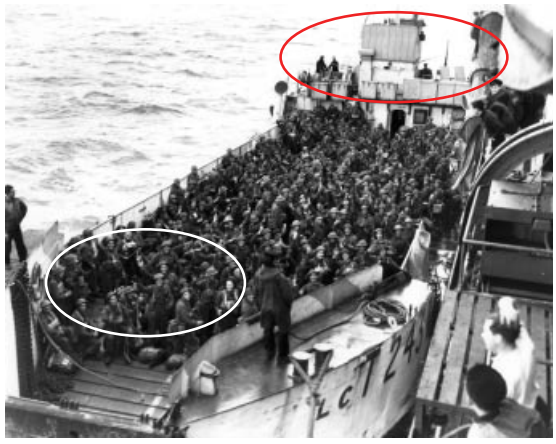


Figure 5.7 This Canadian troop landing ship uses Plan 'B' for carriage of people, namely putting them all together in a single space. The mutiny problem is solved, everyone is equally uncomfortable and anyway there is no choice. Unfortunately, this is not really what is needed in scale, function and durability (i.e. in our terms, not biomimetic of a tissue), e.g. there is short survival time and the space is unlikely to be made larger. Note, there are two groups aboard: crew (red ring) and cargo-troops (white).

two key inputs if we want to keep them usefully functional for any length of time:

(i) feeding; and
(ii) information.

What is more, if these requirements are not supplied, and in good time, the 'cargo' may not just fail to do what is wanted, but may find surprisingly unpleasant things to do instead! In the case of troops, this might be anything from imaginative and unruly behaviour to outright murder and loyalty realignment. Cells can move to the wrong places, build up junk tissues, break down good structures or just die. And of course, we are much better at commanding troops than we are at controlling inherently mutinous cells! In fact, even after we have succeeded in forcing or tempting them to climb aboard our carefully designed support materials, cells have little to encourage them from moving almost anywhere or congregating wherever they want to. In the case of troops on a long, hot voyage, this might be on deck or in the higher, breezy cabins. For cells, it is over the outer surfaces or in the uppermost pores of our constructs.

It is an important similarity that, as with large groups of people, the greater the crush of cells, the harder it is to supply nutrients and oxygen to those in the middle of the crush. In the same way, it is increasingly difficult to get different groups of people in the centre of crowds to respond to complex commands. Again, this is easy to understand for the troops in the landing craft (Figure 5.7). If we give lunch boxes or tea to the men at the edges, it will be a long wait and thin rations for those in the middle of the deck (consumables get consumed!). Equally, if our orders are shouted across the deck, it is increasingly difficult to direct these orders at any particular group of troopers, other than those at the edges. The ones on the middle may not hear, or, worse still, may hear incorrectly above the background.

In contrast, troops on the long-range troop transport ship can have food and drink delivered as it is needed, down corridors and between decks (rather like the way vascular conduits deliver blood). Similarly, information and commands can be targeted in time and space to just those groups of troops who need them. For example, the bridge engineers and sappers might be sent maps of rivers and fences they need to prepare to cross; catering platoons could be supplied with daily cooking or nutrition plans; transport specialists might be given the latest vehicle repair manuals.

Viewed in this way, basic design priorities for 3D construct structures can set as simple delivery vehicles or complex transport systems, depending on the task they are needed for. Quite simply, we need to tailor our cell-landing craft or cell-troop transport vessels to match the application we have in mind.

The first question we need to ask of ourselves, then, is 'what do we expect or hope that our seeded cells will do?' One common answer is that they should leave the support material to migrate out into the tissues and work with the locals (i.e. the patient's cells). This underlies much current stem cell thinking – for example supplying neural stem

cells to treat Parkinson's disease. Alternatively, the idea might be that these cells release growth control factors which will activate or direct local host cells to do a better job, for example cells providing growth factors to promote wound healing. These can be regarded as cell therapies for which the support material is a (short term) delivery vehicle – **a landing craft**.

However, the aim might be to seed in cells which will work away on the support material to form a native 3D graft tissue. These may typically be expected to form organized barrier layers and zones or to lay down a load-bearing bulk of extracellular matrix or form cell-lined channels for blood or axons from the host. In this case we are asking for major cell activity, IN the support material, over extended periods of time. We are now looking at serious internal 3D structure to allow prolonged cell supply with nutrients, materials and individualized detailed instructions. This is the **troop transport ship** concept.

5.4.2 Getting cells onto/into pre-fabricated constructs (the willing and the reluctant)

Getting cells into position on, or into, the support material of choice has occupied a major proportion of the average tissue engineer's time and published output. The **engineer's** approach is to *place* them where we want them to be, as part of the controlled fabrication process. **Biologists**, in contrast, have tended to look for *persuasive* ways to use the cell's own guidance and movement mechanisms. One example approach has been to provide micro-nano topography (grooves, ridges, channels) of a size and shape that cells recognize and react to. Another is to generate diffusible growth factor gradients.

The theoretical aspirations of such bio-inspired approaches have started out high, aiming to place multiple small groups of cells here and there in the 3D space. In practice, however, these have often given way to increasingly modest goals (Text Box 5.2). It has, in fact, become increasingly clear just how much more detail of the basic biology we must understand before these become practical, robust control mechanisms. In truth, these bio-routes have now been reduced to targets which are either depressingly modest or self-defeating in how long they may take to develop.

It is now clear that a great deal of practical effort has been expended in past strategies just to get the cells to attach at all to some of the synthetic polymer substrates. As a result, a 'lowest common denominator' philosophy has come to dominate, which simply aims to grow epithelial cell sheets *over the construct surfaces* or to tempt stromal cells, such as fibroblasts and osteoblasts, to migrate down to deeper parts of the construct. Forming surface epithelial cell sheets can be achieved:

(i) by *pre-growing* a sheet (in 2D culture) over a period of some days, until it is at least a confluent monolayer and, ideally, is differentiating into the required multi-layer specialist structure and can then be laid onto the construct; or, alternatively,
(ii) cells in suspension can be dropped onto, or flowed slowly across, the upper surfaces of our construct, such that as many as possible attach and grow, again eventually forming a continuous sheet.

Getting cells (e.g. stromal, connective tissue cells) into the **deeper, core** or **interstitial** zones of the 3D construct has either been very easy or difficult, depending on the type of scaffold support material used. As we have seen in the last chapter, cell support materials fabricated using harsh, cell-lethal conditions must, consequently, have their cells added at a later stage of assembly. These materials are predominantly the synthetic polymer, ceramic and glass-like materials.

Where we must have such a *separate* cell seeding stage, a range of ingenious methods have been developed. These include simple surface application of cell suspensions, with some cells falling into or being drawn down into the pores. This can be assisted by centrifugation, blotting or controlled flow. Gradual movement of cells down into deeper zones is now commonly promoted by gentle fluid flow (circulating perfusion) around the construct during culture. This flow can help to reduce the

Text Box 5.2 Examples of aspiration versus reality in biological cues to 'engineer' 3D cell distribution

If we expect to direct and energize, for example, growth/migration of axons to re-connect a facial nerve (perhaps as a result of a road traffic or dental surgery accident), then what is needed? First, axons must attach to the available substrate and elongate along the most direct (straightest?) track possible from one end to the other of our implanted construct to bridge the injury. At its best, this might aspire to get axon regeneration to reflect the density and fascicle distribution or branch structure of the original nerve. In reality, topographical and substrate-material guidance is some way away from being this specific or robust, especially *in vivo*. Our present efforts have produced re-growth which looks more like (b) than (a) in Figure 5.8. This is disappointing, as early non-degradable tube conduits achieved much the same results by just confining outgrowth using narrow silicone tubes.

In another example, we aspired to promote rapid micro-vessel in-growth (angiogenesis) to 'feed' our implants by providing an artificial gradient of the angiogenic growth factor VEGF (vascular endothelial cell growth factor). Unfortunately, it turned out that in isolation and at practical dose-levels, VEGF alone produces a very imperfect, leaky, tortuous vessel bed, resembling tumour vessels. As a result, many workers now just seed constructs with free endothelial cells or 'stem-progenitor' cells that may develop into micro-vessel cells. The hope here is that these cells 'know' how to organize themselves into 3D tubes, which *might* then go on to form vessels joined to the host circulation. This is truly modest and not really an engineering-type target.

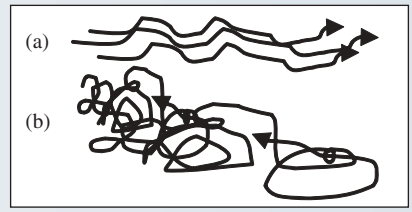


Figure 5.8

sharp diffusion or cell-consumption gradients of nutrients which often form close to the construct surface in culture. However, even modest fluid shear can damage surface cells. The design of such 'pre-fabricated-seed-later' methods tends to be strongly influenced by this driver, sometimes to the exclusion of good 3D structural biomimesis. After all, the presence of 3D deep cells is a defining feature.

In contrast, cell seeding and in-growth need not be major problems for those cell support materials (commonly gels) which can aggregated under physiological conditions. As we have seen already, such hydro-gels are often – though not always – natural materials, and not all of these can be interstitially seeded at the time of fabrication.

Put simply, then, pre-fabricated supports require a separate step for deep cell seeding, while self-assembling materials can come ready-seeded, by interstitial cell enmeshing. The full importance of this difference is another defining feature of extreme tissue engineering (ETE).

5.4.3 Trapping the unwary: Seeding cells into self-assembling, gel-forming materials

As we have seen, once we leave the domain of pre-formed cell supports, our story changes dramatically. Certainly, we must now leave our shipping analogy behind as there are no clear parallels. The trapping of cells into a 3D gel-support material would be a bit like loading a ship by designing it to self-assemble around its cargo – interesting, but *extremely* impractical. In effect, when any one of the gel-forming materials starts to form (e.g. collagen, agarose or fibrin) a physico-chemical change is triggered to initiate monomer aggregation and fluid segregation at a nano-scale (Text Box 5.3). This aggregation almost always involves lateral and very close packing of many hundreds of thousands of molecules. Very close molecular packing is generally essential for the process, as physical proximity promotes the formation of either *many weak* or a *few strong* bonds, all of which are short-range.

Text Box 5.3 Fibre self-assembly into a 3D cell-support gel and the enmeshing of cells

Prior to gel aggregation, we must normally prepare a solution of the monomer molecule. This is a homogeneous dispersion of monomer molecules (collagen, fibrinogen, agarose, etc.), evenly and randomly distributed throughout the water phase (Figure 5.9a: pink represents the aqueous 'shell' surrounding the monomer). In order to understand this better, it is useful if we focus more on the water and less on the monomer.

Each monomer molecule is surrounded by a similar (average) water shell, mainly governed by the monomer : water ratio, i.e. the starting monomer concentration. In the example of collagen fibril aggregation to a gel, this 'ratio' is commonly around 0.2–0.5 per cent monomer, or 99.8–99.5 per cent water – 2 mg/ml protein in water. In this example, each monomer is surrounded by roughly 500 times its own mass of water.

While the term 'hydrogel' should already have tipped us the clue about this water dominance, the *extent* of its huge excess might be a surprise. When gelling is triggered, monomer molecules rapidly pack together side to side into dense 'fibre' aggregates (Figure 5.9(b,c)). Naturally, as monomer molecules pack closer and come into contact, much of the 500-fold water excess must be redistributed. In effect, as the monomers get closer together they push out the water. 'Out' in this case is outside the fibre volume, or into the inter-fibre spaces (pink rings in Figure 5.9d).

The fibres become relatively dehydrated. While the overall ratio of protein to water is the same before and after gelling (still 500:1 – Figure 5.9b to d), its distribution is now non-homogeneous, starkly segregated into solid fibres containing little water, separated by watery zones containing little protein/monomer. The aqueous inter-fibre spaces can be considered as the pores. The gel forms when fibres are sufficiently entangled, or randomly enmeshed, to support transfer of mechanical loads across the material (i.e. to act as a 'solid'). Despite this, the **average ratio of protein to water has not changed** (still 99.8 per cent water).

When cells are present in the pre-gel stage (Figure 5.9b), their membranes prevent protein or water redistribution to or from the cytoplasm, so they are largely passengers in the segregation/gelling process. As a result, cells (or other particles) become passively enmeshed (Figure 5.9e) between load-carrying fibres. This is not to say that embedded cells are 'mechanically independent' of the load-carrying fibre elements. Even non-attached cells will be compressed by deformation of the mesh, and those attached to fibres will be exposed to complex tensional loads, just as they are *in vivo*.

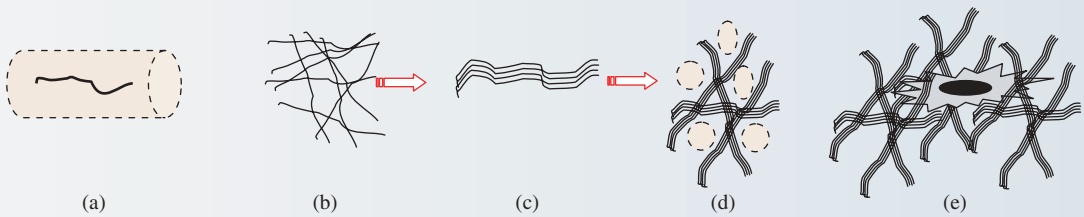


Figure 5.9

As the cells are suspended in a gelling monomer solution and become segregated away from the fibres and into the water-rich fluid phase, they become trapped by the tangle of the newly forming fibres. Scale is key here and, at this stage at least, the fibres are almost always in the nano- or small micron diameter scale (the so-called meso-scale). In the case of our collagen example, fibrils would be ≈30–100 nm diameter, or 150–500 times smaller than living cells (nominal spherical diameter ≈15 μm). We can regard such systems as cells held evenly throughout nano-fibrous networks, surrounded effectively by fluid-filled nano-micro (meso-scale) pores. In this case, cells are physically

trapped and there is no requirement for complex bio-attachment; the cells have no 'choice' (note: to repeat – 'cell-choice' is a human shorthand, not a real cell option). Importantly, this is the environment in which cells live in natural tissues, 'interstitially' distributed throughout the extracellular matrix material. Despite being trapped, they can easily move, either by physically pushing and squeezing between fibres (at this stage, these are very soft gels) or by degrading the protein fibres to form discrete channels, as they do *in vivo*.

Clearly, the gels we are discussing here often have *very* high fluid contents and are, correspondingly, weak. Recent technologies for gel compression and controlled fluid removal have changed this, and offer the real possibility of extending our control of where and how much volume the water occupies. In other words, we no longer have to accept the arbitrary (and high) fluid : cell : fibre ratios which simple gelling leaves us with. We are back in control. These compressed, partly dehydrated gels have all of the biomimetic and cell-entrapment properties of the best extracellular matrix gels, but with the capability to provide usable properties around the cells.

The plastic compression process involves controlled expulsion of excess fluid from between the fibrils by suction or mechanical force (for detail, see Chapter 6, Figure 6.10). In this, the hydrogel, with its interstitial cell population, is subjected to directional fluid expulsion under combinations of compressive load and blotting, typically through a single fluid-leaving surface (FLS). Where fluid extraction is upwards, into a porous plunger, the FLS is at the top, allowing the process to be carried out in conventional multi-well culture dishes. It also means that many layers can be compressed in sequence, on top of the first.

All major parameters of the compressed sheets are then controllable, from the dimensions of the final gel to its collagen and cell density. Each compressed layer is produced in minutes, typically at around 50 to 100 μm in thickness, so multi-layering can be useful. Hybrid layers are simple to form, for example with direct addition of mineral particles, channel formation, incorporation of additional proteins or even synthetic polymer meshes. This strategy provides a new route for accurate engineering of natural materials, comparable with the way that synthetic polymers are made. A core difference, however, is that it is achieved without harsh conditions that can kill resident cells or denature the proteins.

Compression-fabrication of cells into their support material, as described here, is a process that has more in common with biological synthesis than with human industry. It is a bit like building a normal sized house with inflated hollow rubber bricks, then deflating all the bricks so that the house shrinks to doll's house proportions. The advantage is that it is much easier to fabricate the detailed internal 3D structure at the larger scale (with the bricks inflated). Similarly, it is also much simpler to position the desired groups of inhabitants, especially in the deeper rooms, *before* the house is shrunk (deflated). It gets around the compound problems of our need to make precision structures at the **cell scale*** when we are forced to work at the **human scale**, and being at the same time restricted to using difficult, complex building materials under assembly physiological conditions. Taken together, the material and physical restrictions alone make this a tall order. Having the ability to dodge the 'scale problem', with this shrink-compression trick is an essential enabling factor.

* Cell scale is nominally 20μm and human scale is nominally 2,000,000 μm (i.e. 2 metres). This makes one smaller by 5 orders of magnitude, or 100,000 times bigger, depending on your view!

To conclude, then, for pre-formed cell support materials (e.g. synthetic polymers or ceramics, requiring cell seeding) there is a cost benefit tension to be analyzed for each tissue engineering application. We must balance the poor biomimesis inherent in large, shallow cell accumulations and surface-deposition of cells against their advantages of simplicity of production and use (i.e. the basic landing craft analogy). On the other hand, do the requirements of our application demand

that cells are placed into specific locations and patterns, often deep in the 3D structure, where the local environment supplies instructions (bio-cues), nutrients, etc? In the latter case, good biomimesis comes at a cost, namely the expense, complexity and uncertainty of producing natural materials. In some ways, these resemble the trans-oceanic troop transport ships, like SS Aquitania, but their production technologies are not as well understood as those of synthetic materials (Text Box 5.4).

This will need a significant research effort. The first ('landing-craft') option can suffice for quick, minimal-culture applications, where delivery of cells alone is enough for repair. But to engineer functional, 3D *graft* tissues, with prolonged culture, such minimally mimetic supports may not be sufficient. In this case, the 3D structure of natural, interstitially cell-seeded support materials is better, and the traditional problem of high water content/poor mechanics may have been resolved.

5.5 Acquiring our cells: recruiting the enthusiastic or press-ganging the resistant

Where do we get our cells from, once we have decided how and when to cell-seed? This subject is huge. Happily for this chapter, though, much of it is otherwise known as **cell and developmental biology**. Consequently, like so many other core components of tissue engineering, it is knowledge and technology which is accessible from *elsewhere*, as and when needed. The trick for tissue engineers is not necessarily to have an encyclopaedic knowledge of cell biology (that is for cell biologists). Rather, those who need to use this part of the tissue engineering landscape need to understand the location of the solutions. These are the river crossings and mountain passes which can be vital to any successful journey to a tissue engineering application. But most importantly, we first need to ask ourselves *why* we need to cross these hazards at all.

The twin kingdoms of cell and developmental biology are home to some of the most vigorous and dynamic of the tissue engineering tribes. There are innumerable possible combinations of cell types and phenotypic shifts, and such shifts in cell behaviour function are the essence of how embryos develop, wounds repair and, more darkly, how tumours form. These cell shifts comprise the 'mountain ranges' which lie across many tracks of tissue engineering, and down from these comes the torrent of risk and opportunity which we might call *stem-progenitor cell biology*.

In the early stages of 3D culture (see Chapter 3), the concept was to isolate and grow cells directly from the tissue which we needed to regenerate. The main question at that point was whether to use cells from the patient (autologous cells) or from a safe donor (allogeneic cells). Hospital-based initiatives and service industry models have tended to concentrate on autologous cell sourcing. On the other hand, manufacturing industry models aim towards producing reproducible 'packages of bits', off the shelf, for use *now*. These tend to favour using allogeneic cells, but examples of both models are common. More recently, the aspiration has been to prepare early adult stem or progenitor cells from suitable sources and force them down the required cell lineage.

The tension between allogeneic and autologous sources has not disappeared – it has just taken a back seat. The stress has now shifted to trying to supplying *suitable* combinations of biochemical, spatial and mechanical signals, in sequences which fool uncommitted cells to become the cells we want. In common with many targets set by the bio-tribes, we must take careful note of the 'suitable' caveat. It is now very clear that this caveat is shorthand for the need for much more basic knowledge.

We certainly do need to understand stem-progenitor cell controls much better if we aim to use them in an engineering sense. It is also true to say that this topic is not really the *new research question on the block* which it might seem to be. Indeed, there have been determined efforts to tempt bone marrow stromal 'stem' cells to make bone or cartilage since the pioneering work of Friedenstein, Owen, Howlett and others, some decades ago (Text Box 5.5).

Other sources of adult stem/progenitor cells for engineering include adipose tissues (fat – a convenient by-product of liposuction), skeletal muscle,

Text Box 5.4 Micro-porous versus nano-porous cell support scaffolds

Getting cells into their support materials: a story where success is a problem and less can be more.

With a generous slice of hindsight, let us analyze the consequences of having to populate either (i) self-assembly or (ii) pre-fabricated cell support materials with cells. This time the task in hand is to analyze the *primary* consequences, when we must have a separate, specifically designed ***cell-seeding stage***. The 'logic-box' below summarizes why pre-fabricated scaffolds can suffer such poor deep perfusion of nutrients and oxygen. The obvious starting point is that pre-fabricated scaffolds need seeding, but self-assemblers do not.

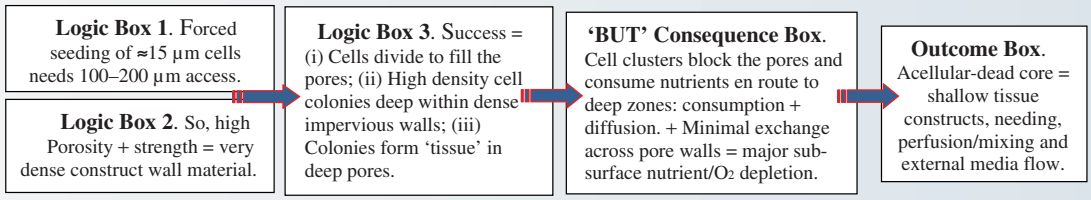


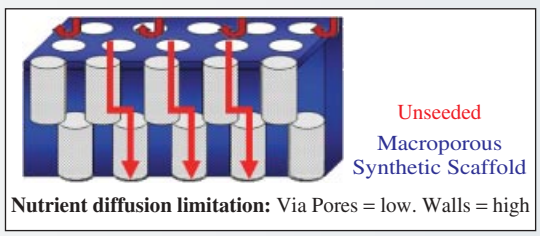
Figure 5.10 below summarizes 'before and after' cell seeding and growth; the more successful this system is in surface cell growth, the greater the problem of poor nutrient access (mass transport) to deep cells. In other words, success brings a fatal problem.

This analysis is completely upturned where we consider self-assembling, cell-enmeshing materials because:

1. a separate, forced cell-seeding stage is not needed (cells are interstitially located at time zero);
2. submicron material pores are around *all* cells as spaces between the matrix nano-fibres, *but*:
3. such nano-pores present minimal diffusion barrier to oxygen, and small nutrients = rapid nutrient mass transport between cells;
4. clustering and 'consumption barriers' only occur at very high cell densities.

Paradoxically, then, nano-fibre self-assembled materials *minimizes* 'barrier forming' tendencies, whereas macro-porous materials promote cell-clustering, path blocking and, thus, poor mass transport to deep cells. In this case, then, *less really is more*!

Tip: The biomimesis of natural nano-fibrous materials may give us clues about how natural bio-systems work. If you have followed this analysis closely, you will realize that such nano-porous materials do not *only* enable mass transport of small nutrients to deep cells. This structure will restrict the movement of macromolecule *products* of cells (e.g. proteins, polysaccharides, etc), depending on molecular radius and inter-fibril spacing. This becomes a cell 'valve system' where access of raw materials to cells is free and non-directional, but export is restricted in rate and direction (by fibre material anisotropy), as it is in most natural tissues.



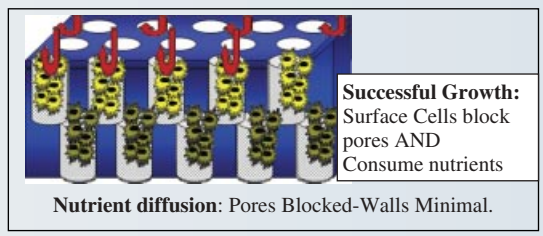


Figure 5.10

Text Box 5.5 Bone marrow stromal stem cells: hot off the press?

Well, not really. Despite the current excitement about the use of adult progenitor cells, especially from bone marrow, for new-tissue-generation, it is actually not such a new trick. Friedenstein *et al.* of Moscow University (1966) were quietly working with others (Owen & Friedenstein, 1998) to understand how to use these same technologies back in the early 1960s – 50 years ago. To give this timescale some context, most family TVs were black and white (monochrome) and powered by heated glass valves! Modestly, these researchers quoted the cell precursor ideas from still further back in time, from Burwell and others.

So the answer to our question is 'no' – generating tissues using marrow stromal stem cells is definitely *not* new.

References

1. Friedenstein, A. J., Piatetzky-Shapiro, I. I. & Petrakova, K. V. (1966). Osteogenesis in transplants of bone marrow cells. *Journal of Embryology & Experimental Morphology* **16**, 381–390.
2. Owen M. & Friedenstein A. J. (1988) Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Foundation Symposium* **136**, 42–60.

cornea and blood. However, in many cases, these cells are vanishingly rare in the overall population or already partially committed to one fate. This can make them difficult in practice to find and expand, or less amenable to multiple uses. The problem is that the signalling systems involved seem to be immensely complex, rather like the patterns of noise, face and hand movements which we humans use for communication. Unfortunately, short of a few crude grunts and shouts, we do not really understand the cell language. While it is clear that an understanding of this biological language *would* have huge value to tissue engineering and regenerative medicine, it also seems premature to consider these attempts as 'engineering'. As an analogy, imagine trying to back-pack across the more remote parts of China knowing only 'xia xia' (thank you).

So, getting hold of the '*cells you want (i)*' (i.e. cell acquisition) is the first target. Then we come to the question of how best to processes these into a '*useful cell preparation (ii)*' and further, what is necessary to push these into exactly '*the type of cells we need*' *(iii)*? The current difficult concept areas (as opposed to technical hurdles) lie in the italics, *(i), (ii) and (iii)* above. Not all of the cell types that we would like to use *want* to be acquired (i), expanded (ii) or differentiated (iii). Sometimes we have volunteers (e.g. bone cells from bone marrow, corneal epithelium from the corneal limbus), but all too often we are dealing with conscripts or, at worst, press-gang material.

For example, we might need cells which produce 'cartilage' – but which type of cartilage? There are different forms of cartilage (e.g. ear, meniscal, articular, growth), some of which grow, some of which do not, and different types will work or not work in different body sites. What makes these different cells different and how likely it is to alter their habits is not well known. Strategies range from taking cells:

(i) from the tissue we want *to make* (hoping they do not change);
(ii) from a similar but more convenient tissue (hoping they change a little); or
(iii) from un-programmed cell populations, hoping they will know exactly what to change into, and do it.

These are the 'same tissue' (volunteers), 'similar tissue' (conscripts) and 'stem cell' (press-ganged) options. It should be alarming, however, to note just how much 'hoping' is going on, and how some of these aspirations are *opposites*.

All too often, of course, the key tension is between the advantages and the difficulties of the acquisition and conversion stages. For example, adult stem/progenitor cells, in principle, seem to have all the advantages in terms of acquisition. They can be

harvested from fat, muscle or bone marrow aspirates with *relatively* ease from many patients, making autologous grafts a possibility. Unfortunately, once acquired, these potentially ideal recruits turn out to need serious pressing, cajoling and training in order to expand their small numbers and to persuade them that they *are* what we *think* they are! All too often, with stem/progenitor cell sources, we find a hugely complex, poorly understood set of problems in driving them down desired differentiation pathways.

This is exemplified by the oldest work in the area – that of getting marrow stromal stem cells to form either bone, cartilage or fibrous tissue cells. This is a problem tackled to date through a host of growth factor and mechanical loading routes. In contrast, mature, differentiated cells, acquired from the target tissue itself, tend to be more numerous and active in expansion. They often are far less ambiguous in their need to be (re)differentiated – tendon, dermal fibroblasts or vascular smooth muscle cells being pretty well what they claim to be on the label. However, these willing troopers-for-the-cause can be hard to acquire, requiring significant lumps of deep tissue to be hacked out, extracted and sifted, through long processes.

What is more, the practicalities of dealing with sick patients can, as always, get in the way here. For example, cells from older donors (a frequent feature) divide very slowly, so need long expansion times. Other significant groups of patients (e.g. cancer) need to take cytotoxic drugs, so their cell division is seriously inhibited. Also, de-differentiation is constantly possible. In the case of cartilage cells, there is a constant tendency for them to drift towards a fibroblast-like phenotype in culture, and so stop making cartilage.

The technical details by which researchers currently attempt these three stages now comprises a large proportion of the conventional TERM literature. It also has, at present, a strong focus on techniques and phenomena rather than concepts and mechanisms, and so is beyond the scope of this work.

5.5.1 From cell expansion to selection and differentiation

Cell expansion is a curious term. At no point is there any intention to produce giant cells, pushing off the culture lids, or to have them straining to open the incubator door. The missing word which makes sense of the phrase is 'population' (after 'cell'). Outside the world of cell technology, it is probably a good idea to make sure the 'population' word is kept firmly in place. This is because the other implied (i.e. unspoken) technical aim is to expand *only* the population we *want*!

Expansion of a cell population, then, requires a technically specialized stage to generate the desired density of the required cell type(s). At the same time, though, there should be the smallest possible increase in the number of contaminating, less desirable cell types. To this end, much equipment and tissue culture processing has been developed, forming a distinct branch of the discipline in itself. However, while much of this technical detail is beyond the scope of this book, one basic message is important. It is, presently, almost always necessary to have a *separate* cell expansion stage. This is because:

- cells tend to be bad at doing 'division' and 'other activities' at the same time; and similarly,
- they also tend to need different growth conditions for fast division than for other activities.

Wherever the cells are acquired from, it is critical for cell therapies to generate enough active cells to carry out the functions that we predict will be needed to make the new tissue. In other words, there needs to be a cell population expansion stage to give sufficient[8] cell numbers for their function. It is commonly assumed that this function would be to 'fabricate' the new tissue. Though there is often rather little mechanistic analysis, the term *sufficient* cell numbers frequently seems to mean as large as economically/ethically possible. Indeed, there have been examples of risky, self-feeding logic in this

[8] Again, the scary bio-caveat '*sufficient* or *suitable*'.

area, suggesting that where a favourite cell therapy does not work as expected, then it just needs more cells to be effective.

However, there is another of our TERM tensions here which it is important to recognize. The thing is that many tissue engineers (mainly the cell-biological tribe) are far more fixed on the importance of getting the 'right cell type' for the job. As a handy analogy, we shall call this the tailor's dilemma.

Legend has it that tailors of fine garments continuously agonize over the relative importance of the quality and the quantity/amount of fabric they use for any given suit ('never mind the quality, feel the width'). In the case of cells for tissue constructs, the argument goes:

- Cells of a *suitable* phenotype (basic behaviour patterns) for the repair/regeneration task in hand are present in tissues because they perform that task in nature.
- It makes sense to use only the best cells for the job, as irrelevant or less effective cell types will just clutter the space, consume nutrients and degrade the process.
- Finally and critically, don't worry about starting cell numbers, as processes in the body often start with a tiny population of key worker-cells which proliferate to give the required numbers naturally.
- After all, cells divide; and the best place for this is in the body!

This, of course, is the 'never mind the width, feel the quality' argument and, on the face of it, it is a potent case. It certainly helps to distinguish the present strategy for the use of stem/progenitor cells. After all, (the argument goes) stem/progenitor cells may be very low in density but they have, in fact, evolved to do just what we want – provide a few cells which locally 'become' (i.e. differentiate into) the cells we need, then proliferate rapidly.

In contrast, (i.e. the 'width-matters' argument) goes: mature cells derived from the target tissue (e.g. cartilage, dermis, tendon) are adapted to *maintain* the fabric of an existing, intact tissue, not to build it from scratch. Indeed, when one smashes up and extracts cells from the simplest of mature tissues, its resident cells are still not a simple homogeneous population that we can 'expand'. For example, there is good cell physiological evidence for at least two metabolically different fibroblast types in skin dermis (and then only because there are two commonly named layers to correlate them with). In normal joint articular cartilage (only 1–2 mm deep and non-vascular), there are perhaps three or four identified chondrocyte types. This increases if we include the meniscal cartilage (a favourite footballer's injury) and aging/degenerating chondrocytes.

What do we get, then, when resident cells are extracted from mature tissues and grown up to expand their numbers in culture? The result of this operation can get complex when cells from associated structures find their way into the culture, from blood vessels (e.g. smooth muscle cells), nerves (perineural fibroblasts) or adjacent and attached tissue layers. Each cell population in these mixed extracts can and *will* proliferate at different rates under standard culture conditions, so after a while one or two types may overgrow the rest while others die out completely (Figure 5.11).

These dynamics need to be understood if we are to devise any rational strategy for the cells we use. For the cell specialist (analogous to the fine tailor, feeling the fabric quality) this requires knowledge of:

- the cells present;
- culture conditions which drive some cells down required 'lineages' (differentiation tracks);
- conditions needed to reduce or kill off those types which are not needed.

For the non-cell specialist tissue engineer, (analogous to the tailor who looks just for the thickest suit fabric), the argument is that this level of control is implausibly complex and difficult to achieve from our present knowledge base. So, pragmatically, (s)he adopts strategies to get as close as possible to the cell type needed in a mixed population. Ultimately this relies on high gross cell numbers to ensure that there are at least *some* useful cells in the final mix.

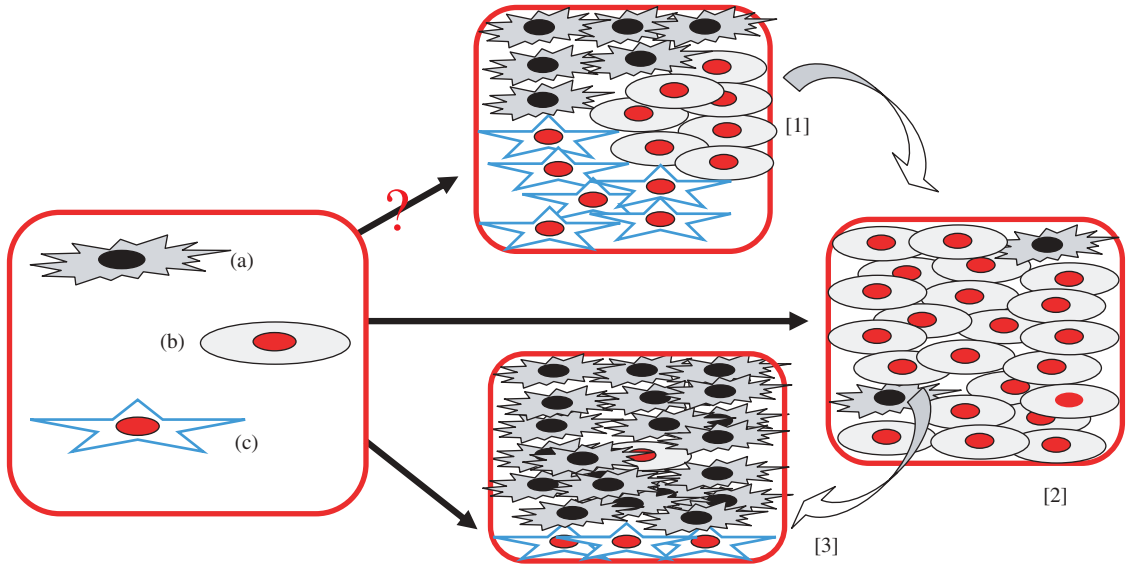


Figure 5.11 Basic strategies for 'cell expansion'. Outcomes range = (1) < (2) OK < (3) A pain. How many cells do we need for our particular application? In engineering systems, this could be a relatively formulaic problem. How much of the required work does each cell perform per hour; what is the total of this work that is needed across the whole tissue volume; what cell density is 'too low'? Unfortunately, we normally are pretty vague about *how* our seeded cells are going to achieve, or even contribute to, the regeneration we want. As if to amplify the problem, the distribution of cell (sub-)types might almost be different for every application. The diagram shows a typical set of unknowns, with (say) three cell types or cell phenotypes in the original population – (a), (b) and (c). [1] These might (at least for one or two passages) divide equally to leave the ratio of cell proportions the same (this is less common). [2] Alternatively, cell type (c) may not divide at all and die out, while (b) divides many times faster than (a), producing a culture with a very high (b) : (a) ratio. [3] Finally, it may be that one cell type (in this case (c)) only grows in one spatial zone of the culture vessel, for example forming an underlying layer. Of course, it is also possible that these culture types can change from one to the other with increasing passage number. It is a brave tissue engineer, then, who predicts precisely how these cultures make tissues in a 3D support material. So, the only safe answer to the question 'How many cells is enough?' (which we cannot know with much certainty) is 'As many as possible', i.e. the 'sufficient' caveat.

This tension between two logics, *refinement-selection* versus *pragmatic bulk*, sums up the present position for cell acquisition and processing in much of regenerative medicine and tissue engineering (Figure 5.11). However, as in the case of our analogy of the tailor's dilemma, it may be that there can, in reality, be no 'correct' or 'high moral ground' resolution. Rather, what we may have described is a necessary working tension.

It is, then, not actually a good idea to aim to 'remove' the tension, but rather to work to resolve it for any given cell type and application. After all, the tailor in the end can ask his customer what the suit is for and make an informed decision based on the *needs and requirements* of the fabric. This is a key phrase. The reader may also have noticed how often it has occurred in the preceding tissue engineering logic. Cell decisions depend almost entirely on what the tissue engineer considers is the *main function* of the seeded cells in constructing the new tissue. Unfortunately, as we have seen many times already, our basic knowledge of tissue repair, regeneration and remodelling is not always sufficiently robust to add this detail. Where such knowledge is absent, or collaboration with the right tribe is missing, we can drift to pseudo-engineering design.

Text Box 5.6 The hidden 'big question': how will we know the cell number that is 'sufficient'?

In engineering systems, this would be a relatively formulaic problem: how much of the required work does each cell perform per hour; what is the total of this work that is needed across the whole tissue volume; what is the net loss-rate of cells; what are the operating ranges of these values?

Sadly, since the biology does not yet allow us to be sure what these seeded cells are doing in any given tissue site, we find ourselves closer to guesses than to predictions. Currently, one of the most common and pragmatic rationales is just to increase the cell seeding density as high as practically possible, on the assumption that*more must be better*. Actually, this can prove to be a dangerous logic (i.e. without a mechanism of action, 'more must be better' cannot be assumed to be true).

As a result, where tissue generation, repair or regeneration fall short of expectations, there is a tendency to use the circular logic that the initial cell density was 'too low'. Consequently, the only safe answer to the question 'How many cells is enough?' is that we cannot know until we stipulate what they do in tissue formation.

The conclusion of this section, then, is that neither a simple 'cell expansion' nor the 'selective cell differentiation' approach can presently be considered a definitive one-size-fits-all answer. In some tissue/repair applications, crude separation and simple expansion of mixed cell populations will be sufficient. In others, much more cell selection and control of differentiation will be needed. The question to ask in order to progress, then, ceases to be which of the two tension-strategies is 'correct' (commonly it will be *neither*). Rather like the tailor's dilemma (see Chapter 7), we must be happy to work with the tension, understand what our cells actually *do* in each specific application and 'tailor' (sorry . . .) the strategy to that. At the same time, it is critical to research the underlying mechanisms of cell tissue repair and remodelling in order to improve our decision-making. In other words, we need to find out just what our cells *really do need to do* (Text Box 5.6).

This is another of our extreme tissue engineering moments where a concept shift emerges. It is inevitably complex, as biological understanding is still developing (sometimes out of engineered tissue models themselves – see Chapter 1). However, to get deeper into this *hunt-for-function* we should turn back to our earlier analogy, comparing cell support materials with troop carriers and landing craft. Look at Figures 5.6 and 5.7 (large troop ship and small landing craft). There are at least two basic functional groups of people being carried: the troops, who will carry out a role on arrival; and the crew, who operate the delivery system, i.e. the ship.

5.6 Cargo, crew or stowaway?

Basically, in the business of maritime transport of people with a job to do – be they troops. plumbers or wind-farm engineers – the ship will carry *at least* two distinct groups. These are the crew, needed to operate the ship/transport, and the workers themselves who do their various jobs once they disembark. In the same way, it is possible to consider two roles for the cells we put into our scaffolds and constructs.

5.6.1 Crew-type cells: helping with the journey

Cells which work on the scaffold during a culture stage or maintain the function of other seeded cells might be considered as analogous to the crew of our transport ship. In constructs that are cultured for substantial periods prior to implantation, this distinction between crew and troops can be an elastic concept. In effect one set of cells are needed now (vessel crew) the others are needed later, so one function can merge into another over time. For example, the fibroblasts which are seeded into the dermal equivalent collagen of Apligraf™ have the early stage function of contracting the loose collagen network down to a denser, tissue-like material before it can usefully implanted.

In some cases, mature, differentiated cells have been seeded into populations of stem/progenitor cells with the intention of enhancing stem cell differentiation towards that cell type. For example, bone cells grown together with endothelial precursor cells will push along the endothelial differentiation to produce spatially defined micro-vessels, as defined by the Kirkpatrick group (see Further Reading). However, chondrocytes or chondrogenic stem/progenitor cells, when seeded into synthetic polymer meshes (e.g. PLGA) can be cultured to lay down the initial rudiments of a cartilage matrix to support repair. In these cases, cells have a crew-like function, helping to prepare the tissue construct before it is implanted. In contrast, there is clearly the separate aspiration that some of the seeded cells will continue to make dermis and cartilage, or link up to host micro-vessels, after implantation. This function, beyond culture (the role of the troops who are carried in our analogy), is intended to help with tissue construction or integration at the implantation site. Cartilage is a special case here, as there is generally little expectation that surrounding, host-tissue cells will be recruited to help, and definitely no neural or vascular in-growth after implantation.

5.6.2 Cargo-type cells: building the bulk tissue

Bone marrow stromal stem cells are often seeded into constructs for bone implantation and regeneration (with or without a 3D pre-culture period). Since this is usually linked to pre-culture with osteo-inductive cues and/or selection to promote osteogenic behaviour of progenitor cells, they are clearly intended to have a bone-building function (and so are cargo-like cells). Experimental attempts to fabricate pulsating heart muscle, using cardiac myocyte-seeded 3D scaffolds, are clearly also intend to carry a cargo of cells with a function in building the new tissue. This is because mature heart muscle is composed of functional fibres, formed from many thousands of such myocytes all merged/fused together into 'syncitia'. Interestingly, most cell-seeding approaches have the default assumption that their cargo of cells work mainly by rebuilding the bulk tissue after implantation. However, as we shall see, this remains an assumption.

Support, protection and integration functions

However, not all cells need to function as construction specialists in the host. Cell cargos can be designed to engineer good integration of new tissue margins with those of the existing surrounding tissues. For example, some researchers have added vascular endothelial cells to their constructs in the hope that these will speed up revascularization/angiogenesis or the in-growth of host vessels from the margins. Similarly, specialist cells called olfactory ensheathing cells have been used to promote and guide neurite regeneration across the margins of spinal cord injuries.

If we stay with our troop-ship parallel, these would be the inevitable platoon of sappers and logistical engineers. Large vessel constructs are frequently pre-seeded with vascular endothelial cells to line/coat the lumen. This is effectively a 'defensive' function, with the intention that such a lining sheet will prevent coagulation In this case, thrombus formation would rapidly block the vessel construct as host blood and blood platelets pass through and contact the thrombogenic wall components, such as collagen or polymer scaffolds.

Concepts surrounding the function of implantable support materials have expanded recently with the wider development of implantable slow- or controlled-release drug depots. Clearly, there is great practical benefit in the addition of relatively common drug agents to assist tissue engineered implants. Examples of this include the addition of antibiotics to skin implants, used in seriously infected wound sites, or anti-coagulants to prevent thrombosis around or in (peri-)vascular implants. Evolution of this branch of refinement research in one direction takes us into the control of release of the drug from the scaffold material so that it works over extended periods. A second branch has been in the binding or trapping of cell-regulating growth factors onto or into the scaffold.

Delivering commands and controls after docking

This track aims to control what the seeded cells do once they are implanted with their support material. Development of growth factor depots within constructs is directly analogous to giving troops on a ship their sealed orders to follow once they are deployed (or in the case of cells, after they leave the lab). Unfortunately, in the case of growth factor depots, it is rarely clear whether these orders are *just* for the troops or for the local resistance fighters (host tissue cells), who will also inevitably come into contact.*

* This assumes we have answered the other hidden 'big question': are we sure we know what our seeded cells need to do, once we have them?

Rather like sealed orders, there can be at least *some* modest confidence that the seeded cells (one's own troops) will take some notice of them. However, what effect they will have on the locals (resident tissue cells) is pretty well anyone's guess. They may not even be in the local dialect. Growth factors on a scaffold can be made stable in the lab, but (like orders when they have been unsealed) they degrade and diffuse away in various unpredictable 'leaky' ways once they get into the tissues. The message-growth factors may conflict with local signals or be unintelligible to the locals when broken down or combined with other orders. So, when the ship lands, or the construct leaves our lab, the chances are that we lose control!

Perhaps you can identify with another analogy; imagine you are a baseball pitcher – in fact, the pitcher in Figure 5.12. The question is, what do you want the ball to do and how can you make best use of your control-window (that is, the period when you are holding the ball)?

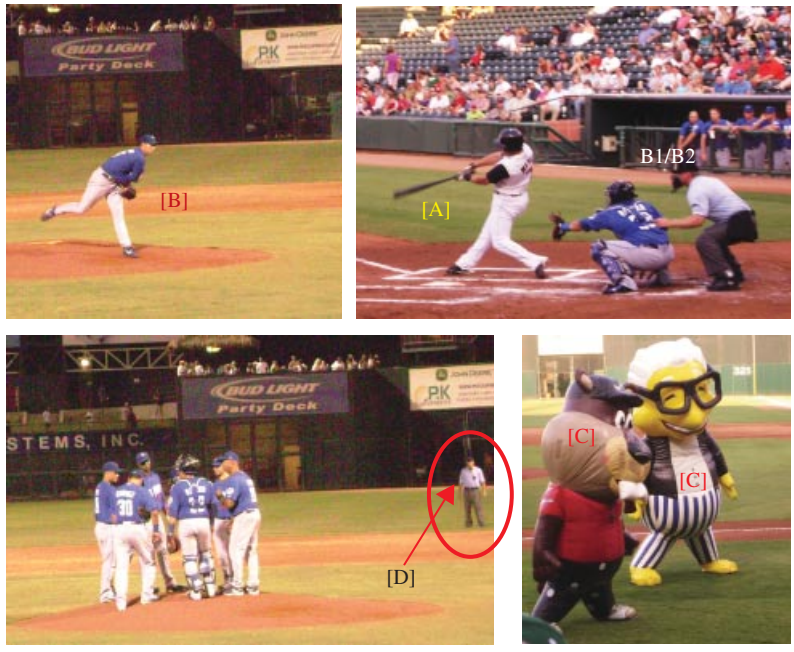


Figure 5.12 Window of control: control of the ball is what the pitcher does (top left) with help from his team (bottom left). But try as they may, once the ball leaves the pitcher's hand, control leaves the blue team. It may go to the batter [A], the catcher [B1] or the umpire [B2] – oops! Alternatively, the latex-wobbly guys might run off with it [C] or it may smack seven sorts of sense out of the sausage salesman [D]. Whatever happens, the pitcher must use all his skill/technology to control the fate of the ball as long as possible *after he lets go*. How would *you* impart post-partum control?

The best we can hope for is a 'window of time' where control can be applied. Unfortunately, this window is typically not just short, but its onset, duration and location can also be almost unknowable.

Simple examples of growth factors delivered to effect tissue responses include transforming growth factor beta 1 (TGFβ1) to speed up extracellular matrix deposition, or vascular endothelial cell growth factor (VEGF) to induce angiogenesis. Clearly, for either of these to be of any help to tissue repair (as opposed to actually doing harm – see Chapter 1), they need to work at specific times and locations. In fact, one of the few points we can be clear on is that when such growth factors go wrong, they can generate tissue scarring and disruption.

However, in another example of extreme tissue engineering, a new generation of approaches may be emerging. The idea here is to trigger specialized depots of seeded cells to generate an *appropriate* full physiological *cocktail* of growth factor signals to stimulate the desired response, at the chosen time and place. An advanced form of this strategy has been described for controlled angiogenesis, or the ingrowth of surrounding blood vessels into the construct or implant site. In this illustration, a very high density of selected (expendable, suicide-squad) cells are positioned as a depot, deep in the construct. Not surprisingly, this dense cell-depot generates its own local hypoxia, inducing local cell stress and even death, but it also elicits release of the full, physiological angiogenic growth factor cascade, just as a local hypoxia would *in vivo*. The result is that surrounding endothelial sprouts invade and form new blood vessels (Figure 5.13).

The key point of this example is that resident cells can be tricked into eliciting *perfectly normal* (i.e.

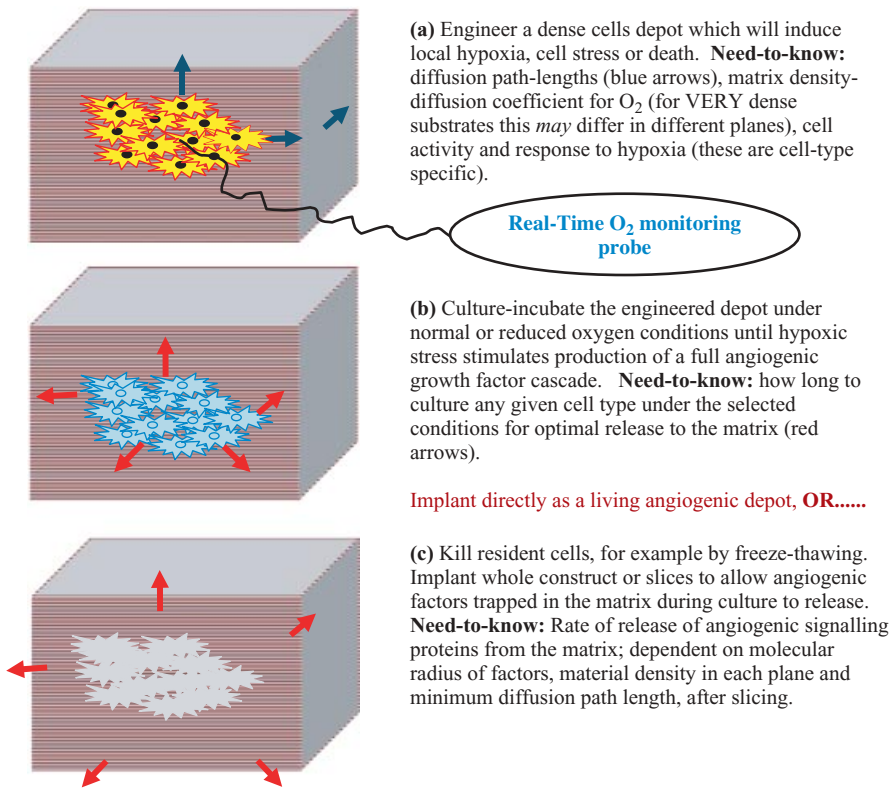


Figure 5.13 Scheme to show engineering of hypoxia-induced angiogenesis. Idea is to provoke a local cell-depot to make itself so hypoxic that it generates a burst of angiogenic factors. In practice there are some key need-to-knows which can only really be measured in a defined 3D cell-specific-model, ideally calibrated in terms of its core O_2.

physiological) cell-cell and tissue-tissue responses which involve whole cascades of growth control factors. This approach does not require detailed knowledge of the content and sequence of the cascade, just how and when it can be elicited. This is where the smart 'tissue engineering' comes in, since it is the definable properties of 3D engineered constructs which puts us in control. In this case, it allows us to dictate when the angiogenic cascade will be produced, and how long and where the growth factors will be released – in effect, where and when the response-window will open.

This particular example is known as engineered, or hypoxia-induced, angiogenesis. In fact, its success is based in availability of a biomimetic 3D model tissue, with its predictable diffusion properties, known diffusion path-lengths and culture periods. This allows us to control post-release cell responses, with only an incomplete understanding of the isolated factors.

It also forms an important illustration of how seeded cells can, intentionally or accidentally, produce key local tissue responses other than those of simple 'tissue building'.

5.6.3 Stowaway or ballast-type cells

Here our people-transporting analogy hits a divergence. In any case of mass transport (maritime or tissue), there is a larger or smaller issue of the stowaway (right down to fare-dodgers on the Underground). This concept cannot properly apply to cells, as they neither choose to ride nor pay a fare. However, there is an analogous problem, which we shall call ballast-cells. These are the cells we tend to ignore (or fail to acknowledge) in the design of 3D tissue constructs. They come along with those we wish to have, *on functional grounds*. We either cannot or choose not to eliminate them from our heterogeneous initial cultures (discussed above).

However, while it is often inconvenient or non-economic to eliminate ballast-cells, it is not good to ignore them when it comes to implant function. Depending on their density, division rate and metabolic habits, they will consume nutrients, oxygen and space, in competition with those cells which do have a designed function. One clear example of this is the engineered angiogenesis described in the previous section. High densities of ballast-cells, for example, will add to hypoxia and unplanned angiogenic growth factor release. While this could be good for skin repair, it is less so in cornea or cartilage. In some cases, they will take on a non-designed function of their own (desirable or not).

Future tissue engineered constructs may, then, need to be pre-analyzed to quantify the effects, not just of cargo and crew cells, but also of any stowaway/ballast-cells. This analysis is increasingly easy using the quantifiable properties of the 3D construct itself as the test-bed model.

5.7 Chapter summary

To conclude, for pre-formed cell support materials (commonly synthetic materials) there is a cost-benefit tension which needs to be worked out for each tissue engineering application. On the one hand, we can tolerate the poor biomimesis inherent in the large, shallow cell accumulations which result from surface deposition of cells onto scaffolds and simple incubation (the basic landing craft). But on the other hand, it is possible to engage the strategy, involving the expense and complexity of systems which provide environmental conditions, suitable to control cell activity throughout a 3D structure (SS *Aquitania*).

There is a third strategy which may become increasingly attractive in this sector, and this is to minimize the culture period and implant the constructs at the earliest possible point. In the self-assembly sector, often using gel-forming biological materials, cells are enmeshed, biomimetically, throughout the fabric of the material (i.e. interstitial seeding, similar to tissues). The penalties come from the very weak mechanical properties of gels (water contents >95 per cent) and limited ability until recently to control either the gelling process or how the material properties are improved with time.

However, we can now conceive to start on the task of biomimetic 3D structures based on engineered native components, e.g. collagen, fibrin and silks.

Further reading

1. Jawad, H. & Brown, R. A. (2012). Meso-scale engineering of collagen as a functional biomaterial. In: Murray, M. (ed.) *Comprehensive Biotechnology* 2nd edition. Elsevier (in press).
   [The idea of engineering materials out of natural proteins, as we make fabrics out of manufactured fibres: bottom-up.]
2. Curtis, A. & Dalby, M. (2009). Cell response to nano-features in biomaterials. In: DiSilvio, L. (ed.) *Cellular Response to Biomaterials*, pp. 429–461 Woodhead Publishing, Cambridge, UK.
   [Review of how topography – surface structure – controls cells: by leaders of the field for three decades.]
3. Lennon, D. P. & Caplan, A. I. (2006). Mesenchymal stem cells for tissue engineering. In: Vunjak-Novakovic, G. & Freshney, R. I. (eds.) *Culture of Cells for Tissue Engineering*, pp. 23–60. John Wiley, New Jersey.
4. Johnstone, B., Yoo, J. & Stewart, M. (2006). Cell sources for cartilage tissue engineering. In: Vunjak-Novakovic, G. & Freshney, R. I. (eds.) *Culture of Cells for Tissue Engineering*, pp. 83–111. John Wiley, New Jersey.
   [References 3 & 4: Two chapters from this practical guide on cell acquisition and growth, with practical examples of problems and solutions.]
5. Panetta, N. J., Gupta, D. M., Kwan, M. D., Wan, D. C., Commons, G. W. & Longaker, M. T. (2009). Tissue harvest by means of suction-assisted or third-generation ultrasound-assisted lipoaspiration has no effect on osteogenic potential of human adipose-derived stromal cells. *Plastic and Reconstructive Surgery* **124**, 65–73.
   [Modern example of cell acquisition – and such a mind-boggling title!]
6. Ghanaati, S., Unger, R. E., Webber, M. J., Barbeck, M., Orth, C., Kirkpatrick, J. A., Booms, P., Motta, A., Migliaresi, C., Sader, R. A. & Kirkpatrick, C. J. (2011). Scaffold vascularization in vivo driven by primary human osteoblasts in concert with host inflammatory cells. *Biomaterials* **201**(32) 8150–8160.
7. Brochhausen, C., Lehmann, M., Halstenberg, S., Meurer, A., Klaus, G. & Kirkpatrick, C. J. (2009). Signalling molecules and growth factors for tissue engineering of cartilage – what can we learn from the growth plate? *Journal of Tissue Engineering and Regenerative Medicine* **3**, 416–429.
   [References 6 & 7: Taking hints from nature for combinations of cell types and growth factors in scaffolds to mimic development in 3D.]
8. Hutmacher, D., Woodfield, T., Dalton, P. & Lewis, J. (2008). Scaffold design and fabrication. In: van Blitterswijk, C. A. (ed.) *Tissue Engineering*, pp. 403–454. Academic Press, London.
   [Review of conventional theory and practice in current use for biomaterials design in tissue engineering.]