Bioreactor origins

Although 'the bioreactor' has been a major part of tissue engineering thinking and aspiration since its beginnings, it cannot be claimed as special to the subject. Fermentation bioreactors predate us by quite a margin and, if we are to borrow their name, the least we can do is to understand what it *already* means. The simple wine-beer fermentation jar shown here is a classic – if simple – bioreactor in which biological organisms (yeast, in this case) are fed with sugar and other nutrients under controlled, often dynamic, conditions to promote production of useful materials, such as alcoholic drinks. Classically, this involves biochemical reactions and changes, (i) mostly *in solution*, (ii) mostly carried out by *whole organisms*. Interestingly, neither of these features ranks high in the 3D tissue-bioreactors now envisaged for the growth of engineered tissues.

8 Bioreactors and All That Bio-Engineering Jazz

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

8.1 What *are* 'tissue bioreactors' and why do we need them?

The central place of 'the bioreactor' in traditional tissue engineering thinking is suspiciously elastic and ambiguous. The idea of the bioreactor is that the basic building-blocks of the tissue, once assembled, can be brought together into a 3D tissue facsimile by a culture stage. However, these variables seem to have worryingly large ranges, requiring anything from tight cell control and brief culture periods down to those supplying only minimal cell controls over long culture periods. Consequently, the complexity, duration and even the timing of this *bioreactor culture stage* has been difficult to define and understand.

In this case, to be suitably analytical, we should ask: 'Can we be sure that *tissue bioreactors* really are likely to do all that is being asked, or is this a tissue engineering 'fig-leaf' used to conceal an embarrassing area of uncertainty?'

8.1.1 Rumblings of unease in the smaller communities

To start to answer this question (as in other chapters), let us look more closely at some of the really fundamental assumptions of the 'tissue bioreactor story'. Indeed, there is a huge assumption buried deep within the original blueprints for tissue engineering. For shorthand, we can call it the 'architecture-control assumption', which proposes that our bioreactor conditions will control the tissue micro-architecture of the constructs we grow. It now seems that most of us have, at one time or another, signed up to this assumption without really checking the small print. And the small print, not surprisingly, includes a sub-section which states '. . . but do remember, this is an assumption!'

To recap, the assumption is that: 'given a suitable array of control cues and raw materials, the cells we grow will make a new and functional tissue where there previously was none' (Text Box 8.1; also discussed in Chapter 7).

The optimistic view, that this is a safe assumption, comes from:

- Long-held developmental biology theory, and its understanding of how embryonic tissues come together from small cell-balls and
- Some epithelial, sheet-cell types, which can self, assemble themselves into simple but reasonably faithful tissue-layer replicas.

In the same vein, we can see that there has been a significant expansion in structural complexity which can be generated as we moved from '2D' to '3D' culture systems.

In this climate, it is easy to understand the buy-in of much of the community, especially 3D biomaterials scientists, cell and developmental biologists and engineers. However, one of the smaller tribes of tissue engineering has consistently shuffled their feet and kept returning to the 'assumption' word. These are the tissue repair biologists.

Repair biologists (including some surgeons) are also impressed by this vision and they are just as keen for it to become possible. However, they have also been suturing, pouring and pushing every conceivable potion and composition into tissue injury sites that 'most people do not want to know about' for the best part of 2.5 millennia.

Text Box 8.1 Two types of cell-control cue

Viewed from a process technology standpoint, cell control cues here fall into two broad categories. The first type of cue regulates all things related to the composition of the fabricated tissue, including the order, rate and concentration in which components are incorporated. The 'components' will include cell types (with proliferation and differentiation) and soluble and insoluble (e.g. extracellular matrix) export products. This is complex but familiar, especially to the cell biology and medical communities.

However, the second family is far less familiar. These are cues which are needed to control the 'where' – the spatial/temporal organisation cues critical for *functional* 3D architecture.

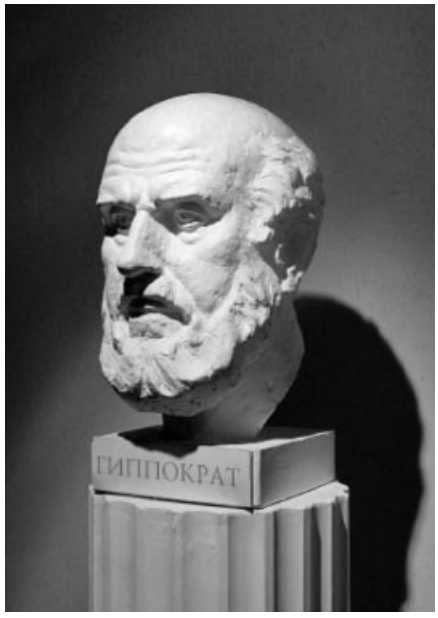


Figure 8.1 Hippocrates of Kos (*ca.* 460 BC – *ca.* 370 BC; Greek: Ἱπποκράτης; Hippokrátēs).
© iStockphoto.com/Philip Sigin-Lavdanski.

Indeed, Hippocrates (Figure 8.1) did not seem to be averse to filling tissue voids with a range of exotic materials. But despite every possible motivation (and surface wounds are *extremely* emotive) and plenty of imagination, success has been strictly modest in getting usable new tissue to form where and when required. This rather does suggest that the 'architecture-control' assumption might not be quite as sound as we would like. Indeed, even where inspirational approaches (including bee-fluids or tropical tree-bark) have been replaced by modern gene and cellular mechanistic therapies, this unease persists.

As discussed in Chapter 1, significantly sized (non-fatal) defects in vertebrates normally fill with tissues which are rarely as functional as we would like. Scars appear in almost every body location. So the repair-biologists logic goes, if molecular and cellular cues *in the correct body-site* cannot, after many millennia of evolution, persuade natural systems to rebuild tissue as it once was (i.e. regeneration, see Chapter 1), what makes us think that farming them in a laboratory bioreactor will do any better? The suspicion here is that the enabling environment of at least some clean wound-beds (post 50 million years of evolution) should be better than a reaction chamber (evolution time ≈15 years). Looked at from a philosophical perspective, the ability to make omelettes with *random structure* out of highly *structured and symmetrical* eggs implies nothing about the feasibility of the reverse process (Dumpty *et al.*, 1835)[18].

8.1.2 Hunting for special cells or special cues

The assumption that our technology is up to the task of persuading cells to do just what we want is a difficult one to break out from. Figure 8.2 shows a logic gradient along which biotech scientists can move freely, working diligently towards whichever end their vision takes them. The logic suggests that we can produce our tissues either by:

1. prodding our rather everyday cells into the right action with subtle environmental instructions/cues; or
2. finding those special precursor cells (which currently seem to exist in embryos) which can produce tissues on their own with very little help from us (a recent sub-plot to this suggests that we can go one step further back, de-programming adult-committed cells, then reprogramming them to something else – in other words, inducible programmable stem cells).

On one hand (a), this implies a breakthrough in the understanding of how to control normal cellar processes, especially 3D spatial controls. On the other (b), it hopes that, through much trawling and good fortune, we will locate cell types needing little or no external control. Clearly, either of these is dangerously open-ended, but the availability of both options apparently makes it possible to offset (or to 'fudge') the risks. In effect, as one approach

[18]Note: this is a joke reference. Please do not be tempted to look it up. It refers to an old British Nursery rhyme about the futility of trying to repair broken eggs . . .

Figure 8.2 Logic spectrum for how to achieve the fabrication of functional tissues through culturing of cells.

hits its inevitable problems, we can move our effort to the other.

However, justification for labouring away at the problem (either end of the logic gradient) is only reasonable where there are plausible approaches or new knowledge with which to assault that problem. The idea of just 'trying' every available version or combination is at best risky, and at worst futile. Just like Hippocrates, tissue repair biologists have been here before – fishing for solutions too far ahead of their understanding. They are now rightly cautious about committing to the same approach for the next 2.5 millennia. After all, although the solution may be only around the corner, scarless healing is *still* a dream.

8.1.3 Farming – culture or engineered fabrication

Although the need for tissue bioreactors in this scheme (Figure 8.2) rises (left) and falls (right), the underlying assumption at both ends of the logic is that cells will be the primary producers of both tissue substance *and* structure. The only remaining question is who (or what) supplies the controls that regulate production rate/sequence, component type and spatial accumulation of material. This reflects the traditional biological belief that only cells can make complex bio-systems. It assumes that the role of engineering systems in the process will be restricted to monitoring instruments, nifty labour-saving culture chambers and cryo-storage. In effect, then, current tissue engineering has an implied assumption that tissue will be made through ever-better cell 'farming' (like salmon farming), rather than engineered fabrication (like mobile phone production).

The role of new science in generating useful products from the culture of living systems has a long and pragmatic history. We can trace this progress from the explosion in agricultural production, ranging from GM products, marine farming, hydroponics and enhanced animal welfare, to safe product storage, cryopreservation and meat traceability. Our analogy here, then, is agricultural.

Despite a long history of scientific benefits in agriculture, many in tissue engineering may not appreciate the drawing of this parallel, yet it is all too valid, as we can see from the evolution of conventional bioreactors. For example, the expansion of cells (especially stem cells) for regenerative medicine involves their growth (proliferation) in nutrient media. This is directly analogous to hydroponic culture of plants or aquatic farming of fish fry in hatcheries (also known as aqua-culture). The aim in each case is to develop defined nutrients, blended with control/stimulant and anti-microbial agents within tightly controlled protocols of temperature, pH, sunlight, etc. These are designed to produce reproducible biological products, from plants or plant products to young fish and fish fillets (Figure 8.3).

Success is, in part, limited by the complexity of the biological system (i.e. the whole organism and the material we intend to produce). In the case of farmed salmon fry (or, later, their maturation into adults), the organism itself holds all the necessary information for production and control to generate new fry, subject to basic conditions such as water content, flow/mixing, temperature and light quality. So, for whole-organism culture (such as salmon), the complexity of the organism and the fabrication controls are not an issue for us as the organism itself comes comprehensively equipped to carry out the *full* process where suitable *enabling* conditions are provided.

In the same way, stem cell expansion, though complex in its detail, is simplified by the fact that the cells themselves come ready-programmed to divide without too much outside control. Clearly, there are questions (particularly for stem cell expansion) of

Figure 8.3 Farmed fish fry. © Gwynnbrook Farm

maintaining a particular differentiation state of the cells. But this is much the same as maintaining good inbred salmon stock over a number of salmon generations despite mutation, genetic drift or infection. In other words, we tend to be generally happy with what the bio-system will produce for us. Much of agricultural science and cell culture, then, is about adapting or enhancing the enabling conditions in order to shift or speed up what we get as a crop towards what we need.

This may seem like a small, useless fragment of pedantic logic shuffling around the similarities of culture and farming, but it is really far more. Once we accept that our concepts have shifted from construct *engineering* (i.e. fabrication) to cell-dependant culture or farming, the more astute reader will see that we are also expecting to break one of the most basic rules of farming. Although the retail value of fish lips may, in some countries, be attractively high, and pumpkin fruit is the only saleable part of that plant, no one is yet suggesting that we farm *just* lips or *only* the fruit (Figure 8.4). The bone-filled fish and the tough leaves are not essential as a product, but they are as much a part of the production process (the culture) as the bioreactor is in tissue engineering. They are not part of the high-value product, but equally there is no product without them.

The complex 3D tissues that we require here cannot be made in isolation, without the control and production machinery. This makes our dream of the tissue bioreactor a deceptively high hurdle, as we have set ourselves, for the first time, the task of producing meat without bone or brain, or

Figure 8.4 'Isolated farm produce'. Pan-ready fish lips (left) and leaf-free pumpkin (right). Clearly, these are agricultural fantasies. We farm *whole organisms*.

fruit without root or leaf. This is the equivalent of a cow-free steak farm – an idea we shall need to revisit.

At this point, it is possible to hear the distant sounds of all-out rebellion from the host of tissue engineers who are keen to assert that culture bioreactors ARE the answer and, in time, we shall find the right conditions. We can in time devise advanced tissue bioreactor technologies which can replace all the missing cues and controls. After all, it is early days yet, as Hippocrates of Kos might have claimed. However, as a result of the **Hippocrates caveat**, it might be wise to run this aspiration through a reality check. To analyze how well this stacks up, we need to look under the lid of 'the history and origins of tissue bioreactors'.

8.2 Bioreactors: origins of tissue bioreactor logic, and its problems

8.2.1 What have tissue engineers ever done for bioreactor technology?

Mammalian cell biologists are sometimes surprised to find that 'bioreactor' is a word which engineers and bio-process chemists commonly consider their own. Rather inconsiderately (for us), we must admit that *tissue* engineers did not invent '**bioreactors**'. This is a term used for many decades (indeed, centuries in the case of fermentation) to describe vessels, frequently stirred, in which suspended cells – from yeast and bacteria to genetically modified mammalian cells – grow and produce materials that we want. Clearly (and happily), the earliest examples of these were the wine and beer products of alcohol brewing chambers. Their modern equivalents now churn out (literally, in the case of milk-derived products) everything from washing powder enzymes to medical antibodies, all with exceptional efficiency and precision.

So, the only special contribution that tissue engineers seem to have made is to adapt this to the culture of 3D (i.e. multi-cellular, spatially organized) structures. In other words, the most important difference is that the product of tissue-bioreactors is intended to be a complex 3D structure, whereas conventional bioreactors produce soluble molecular mixtures. In fact, we can now see that conventional biochemical bioreactors have been developed into highly effective production units in which complex, sophisticated product mixtures are made with 'engineering precision'. While fermentation bioreactors can make highly complex chemical mixes such as fine wines, and perfectly reproducible antibodies genetically modified cells, these products are essentially *soluble*. They certainly have minimal to zero 3D spatial organisation across length-scales greater than a few nanometres – in other words, emphatically *not* what we need for tissues (see Chapter 5).

So, what are the similarities between tissue bioreactors and their conventional relatives? In both types of bioreactors, the function is to 'control biological output' from large numbers of cells, so they both ultimately depend on getting cells to generate product. This culture-dependent production can sit anywhere along a spectrum which runs from biochemical engineering (closely controlled process stages) to more traditional, farming-style culture systems. In the latter case, controls are light and aimed chiefly at enhancing the native biological processes that are inbuilt in the farmed organism. The more controls are applied to such systems as environmental factors, the closer they become to (bio)chemical engineering processes. However, the further down the engineering line the process goes, the louder is the demand for detailed knowledge of the innate cellular controls.

As we move away from systems that depend on good Pilsner beer yeasts and towards those where the producer-cells wall themselves into collagen-elastin tombs (i.e. into a matrix-rich tissue), this detailed knowledge rapidly becomes the limiting factor. Here we reach the big difference. While many decades of research have made it possible to control intracellular processing to produce refined *soluble* products, we are a long way yet from the same understanding where the cell product is a spatially organized 3D tissue (Figure 8.5).

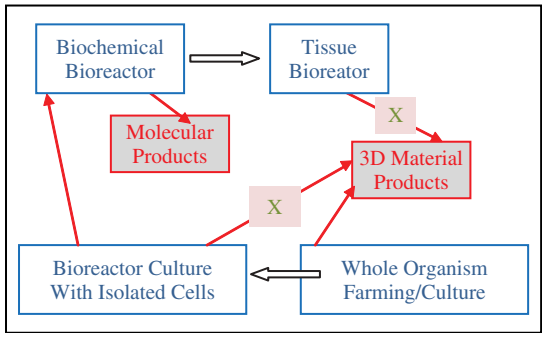


Figure 8.5 Illustration of the bioreactor logic-loop which needs either engineering-level control of material synthesis (not achieved yet) or cultivation-type fabrication without whole organisms (cell-only; again not really achieved yet).

8.2.2 The 3D caveat

Oddly enough, although the transition from biochemical to tissue engineering bioreactor may sound modest, it unfortunately contains a critical caveat which changes everything. This is buried in the quantitative detail and can be glimpsed from two directions. In the first view, we can see that we have quietly moved a long way from the target of making soluble (definitely non-3D, non-material) products. However, our assumption has been that this can (easily) be extended to the *solid* 3D structures required from tissue engineering bioreactors.

Worse still, these 3D structures are not simple to form or easy to maintain using living cells. Target tissues are frequently large in overall dimensions and made of dense material. This leads to inherently poor perfusion (mass transport) properties, especially for the deeper layers. Put another way (but the same caveat, really) in tissue bioreactors the solid material product is deposited like prison walls around the cells which produce it, rather than being pushed out into solution and swept away by the stirrer. This change means that, for success, the quantitatively dominant product will be the bulk *solid* support matrix which makes up the tissue. In matrix-rich constructs (skin, tendon, vessels), this commonly takes the form of new extracellular matrix material (see Chapter 3). In cell-rich constructs, the product is more cells, but in the same structural mass. This is the *prison wall* caveat

However gently we try to pass over the prison wall caveat, its effect on the bioreactor logic is thunderous. By taking the option that *cells will make what we need*, this logic forces us to a heavy dependence on inbuilt biological responses and controls. These are the innate controls of the producer elements (i.e. the cells). But without a detailed understanding of how they operate, our tissue bioreactor aspirations begin to look a very, very long way from our comfort zone of conventional biochemical bioreactors.

Text Box 8.2 Between a rock and a hard place

The whole idea of tissue bioreactors seems to lie precisely at the impact point of a rock-like assumption and a caveat hard place. Could it be that we have here an attractive concept which is shockingly far ahead of its time? The prison wall caveat is that the cell-product *must* end up being an organized, substantial 3D material (i.e. a tissue). This makes the science and engineering needed to control production extremely difficult to understand. The daunting scientific problem, in turn, forces ever greater reliance on cell-dependent (farming-like) processing.

However, as if to lock the trap, we find that such approaches are traditionally only effective where they 'culture' (farm) whole organisms which include a *complete* control system (the fish-lips conundrum). Our only major success so far in fabricating useful products from *part*-organisms or cell-masses lies in the use of conventional engineering bioreactors, which generate soluble, non-material products.

Figures 8.5 and 8.6 illustrate this rather irritating logic-loop which bogs down our dream. From where we stand now, either route out of this paradox would seem to require decades of basic research, not **system optimization**, as often suggested. While this is not a universally welcome analysis, it should deflect us from futile cycles into more balanced, coherent strategies.

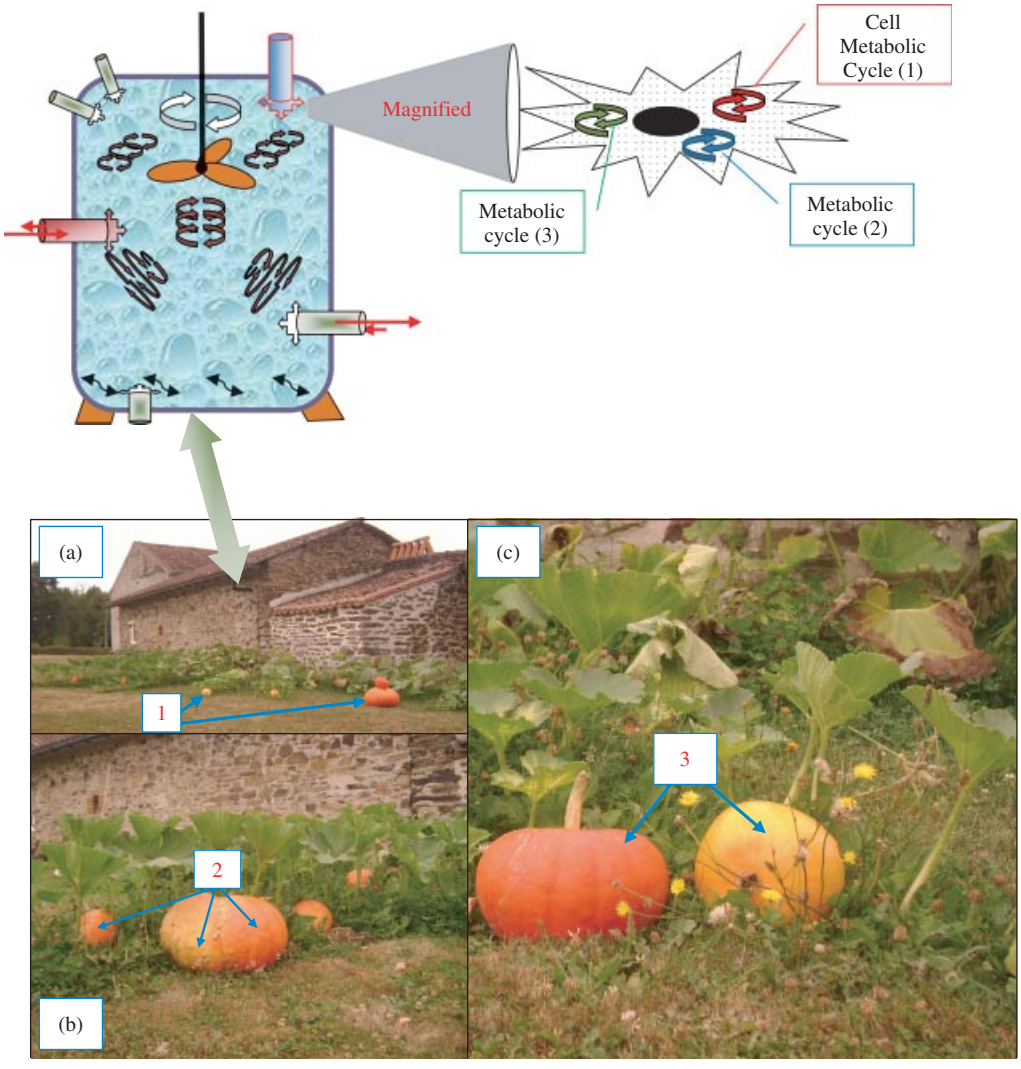


Figure 8.6 Diagram illustrating (by exaggeration) the extremes of culture control, which form the 'bioreactor spectrum'. The top panel shows a diagrammatic form of engineering bioreactor for tight biochemical control of single-cell-suspension production of useful soluble products (e.g. protein). The cell-medium suspension is stirred (local mixing/stagnation/turbulence can be modelled mathematically) for controlled nutrient/gas exchange with cells. Input of metabolites, nutrients, etc., as well as out-take of product and wastes, can be batched or continuous, under tight control. Conditions are monitored (e.g. pH, ionic strength, temperature) for feedback correction. Meanwhile, the main biochemical cycles and enzyme efficiencies (right hand panel; cell cycles 1–3) are known in detail and can be controlled. The lower panel (a to c), in contrast, shows a simple agricultural culture system for production of pumpkin vegetable *material* in large, useful 3D lumps. Though simple, the culture system still supplies essential nutrients and microenvironment (as far as the requirements are known). It monitors basic changes in conditions and reacts to correct these as they develop. However, the precise demands of the system are not (cannot be?) well understood, so the system is based primarily on reliance on the innate ability of pumpkin plants (i.e. the whole organism) to fabricate pumpkins. The controls are very light but, equally, heterogeneities and variance in the system and the product are easily tolerated (arrows 1 to 3).

8.2.3 Fundamental difference between biochemical and tissue bioreactors: 3D solid material fabrication

In effect, all of the tight control and understanding which goes into conventionally engineered bioreactors can be regarded as manipulation of the organic and physical chemistry of two *soluble* compartments, the first is the intracellular cytosol; the second is the extracellular culture medium. Nutrients, gases, waste metabolites, catalytic enzymes and product/by-products/contaminants generated by the cells are in solution, either inside or outside the cell, in the culture medium. This is commonly acknowledged by the idea that 'pools' of this or that metabolite are located within intra- or extra-cellular compartments. There is 'traffic' between pools with characteristic dynamics and rate constants.

This reflects the sophisticated level of understanding of intracellular biochemistry, with its many well-mapped metabolic pathways, predictable kinetics and enzyme control points. This is so much so that the chemical processing can be mathematically modelled down to the level where stirred, sluggish and turbulent flow of fluids can be used to regulate mass transport of products between different parts of the system (extracellular/intracellular pools, compartments or zones of the bioreactor.

Figure 8.7a illustrates this predictability, based on dynamic (bio)chemical processing of solutes in closed chambers, These have characteristically well understood, separated pools of reactants, within a *two-compartment system*. Unfortunately, it is completely unreasonable at present to expect this level of

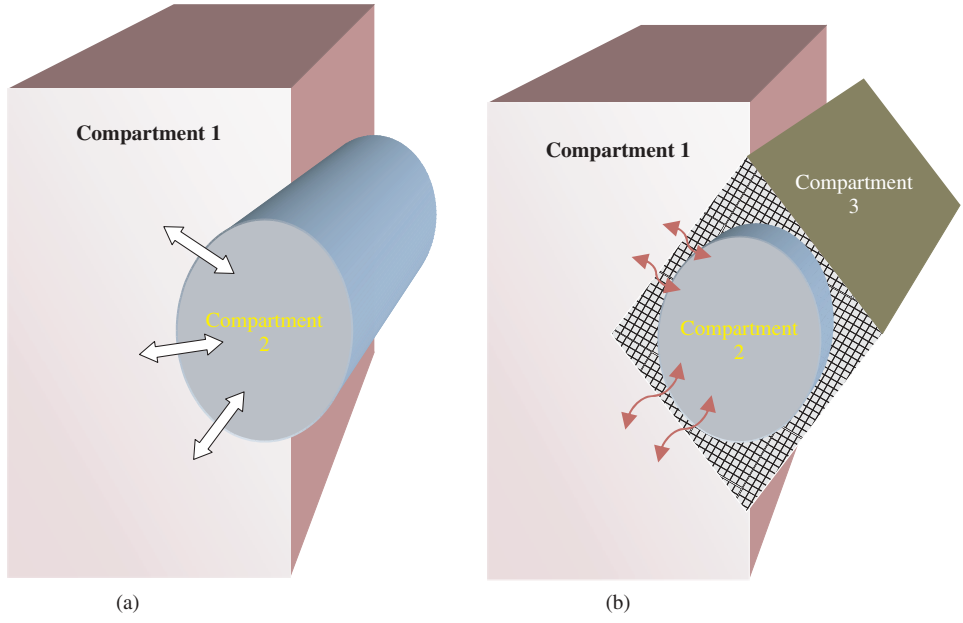


Figure 8.7 (a) Cartoon illustrating a principle of conventional, biochemical engineering, bioreactors. These can be seen as two compartment systems – intracellular and extracellular fluids – separated by the cell membrane. The equations for mass transport of reactants and various cell products can be calculated on assumptions based on access between the two compartments, with mixing or stagnation influencing diffusion times in each. (b) With the addition of a third compartment (extracellular matrix, ECM material) enclosing the cell-compartment, all of those equations are void. First, the third compartment/ECM changes *not only* the mass transport, but its predictability as ECM structure is dynamic, anisotropic and heterogeneous. Second, the main bulk product of the cell chemistry goes into the ECM (so out of solution), altering the chemical equilibria in unknown, relatively unpredictable ways.

control in 3D tissue bioreactors, because the main products, by definition, cannot be soluble. In order to become a solid tissue, the cell products from tissue bioreactors must be packed, in great bulk and at high densities, between the producer cells.

This means that the knowledge base and conventional bioreactor rules, no matter how successful, cannot be applied, because we have slipped gently into what is now a *three-compartment system* (Figure 8.7b). There are, as before, the intracellular and extracellular (medium) compartments with their distinctive, intermeshing chemistries, but physically *between* them is an extracellular (tissue matrix) material – a solid. This changes everything. Not only do the main products *not* mix, traffic or diffuse according to any of the previous rules, but this build-up of solid product increasingly dominates mass transport in the *other* two compartments. Worse still, it does not seem likely that we will ever side-step this third compartment solid matrix 'problem', as it represents the very thing we want to make. This is one issue we *have* to fix.

Far from being a pedantic piece of classification, this understanding is critical. It means that the 'bioreactor' concept is unlikely to get us off the equally difficult farming process problem of expecting to grow isolated tissues without the whole organism (the fish-lip conundrum). Is it possible, then, that tissue engineering has inadvertently set itself an enormously high target by mixing its exemplars? This seems to be a fatal concoction of:

- top-down culture (farming) of isolated tissue-parts, *but* without the luxury, of the entire organism; and
- bottom-up bioreactor synthesis, *but* with the previously unattainable target of controlled 3D architecture.

The difficulty is that the 'but' caveat in both examples makes that route look worryingly implausible.

This, perhaps, finally nails down the niggling feeling from Section 8.1 that we are being held back by a questionable assumption (the Hippocrates caveat). It comes down to our old friend '3D architecture and how we make or grow it'. Next, we should look at the special consequences and needs that develop from this implausibility, which perhaps might allow us to creep up on some new (extreme TE) solutions.

8.2.4 Why should a little thing like 'matrix' change so much?

Predictable biochemical reactions

Those readers who have experience of organic and biochemical processing will understand the dominating importance of:

(a) reaction equilibria on rates of production/consumption; and
(b) mass transport of reactants and products (for large or dense systems).

At almost all stages, the important metabolic enzymes will drive reactions at rates which relate to the concentration of the reactants and products *in solution*. But there's the rub: *in solution*. When a dominant proportion of the soluble cell product *leaves* solution and becomes solid, as extracellular matrix, it ceases to play the same predictable games, and new rules apply (Figure 8.7). Where a reaction product is taken out of the system by leaving solution, we might (simplistically) expect the reaction to be accelerated. There are many simple examples of this in conventional chemistry. For example, where the product of a slow reaction is poorly soluble and precipitates, this loss of product from the soluble phase can pull the reaction faster, due to the removal of product inhibition.

However, the protein-polysaccharide composite materials which make up the ECM considered here are *not* low-solubility salts with known solubility coefficients. They aggregate through complex, time-dependent bond formation, following cell processing, intermolecular recognition and cross-linking. Just for good measure, many undergo maturation through water exclusion.

These processes are currently not well understood, and are certainly not often predictable at a mathematical level. This is especially true for ECM, which is an inherently heterogeneous asymmetrical

material in 3D space. This is not to say that these reactions and production processes will not happen – just that they are transformed from simple and predictable into 'too complex to predict with current technologies'. To coin a phrase to sum up this position: *no predictability, no controlled processing* (or the '**NoPre-NoCon**' principle if you need a mnemonic).

Predictable mass transport of reactants in the 3D space between compartments

In terms of the effects on mass transport, it is important to recall that:

(a) the ECM is the *bulk* material to be produced;
(b) the aim is to promote ECM deposition *between* the producing cells;
(c) good mimics of native ECM will be fibre-anisotropic, with locally distinct zones and layers.

This represents almost the opposite of the separating structure in the two-compartment system – that is, the cell membrane. Cell membrane is ultra-thin and biochemically constant in 3D space and in time – a paradigm of predictability. Any local in-homogeneities of extracellular culture fluid – for example between stirred and stagnant zones in Figure 8.7a – can be monitored, controlled and predicted.

The number one purpose of *tissue* bioreactors is to increase the mass and heterogeneity of ECM material, with properties which are completely different to cell membranes. So the drift (or should that be 'headlong dive'?) away from 'good bioreactor' conditions (predictability of nutrient/waste and product transport) is both inevitable and progressive. The 'progressive' point grows from our requirement that the tissue structure should get more and more complex (i.e. tissue-like).

The important take-home message here is that, on this analysis, the longer our bioreactors are cultured for, the less predictably they will work! Hence, suggestions that our bioreactors will give us the tissues we need if we could just run them longer and longer sound like ever-bolder strides out into the valley of death (complete with whistling a jolly tune). Since we often do not understand the timing of cell-mediated ECM deposition, we cannot yet hope to formulate the new equations that might lead us forward safely. Once again, this is not to say that these processes cannot occur to make ECM and tissues in bioreactors – clearly, they do. It is just that two-compartment thinking cannot be used to *predict* the process (and **NoPre-NoCon**).

8.2.5 The place of tissue bioreactors in tissue engineering logic: what happened to all the good analogies?

We might expect that a good analogy of the fabrication (engineering type) systems considered here could be the manufacture and assembly of cars. Basically, the process sequence resembles our tissue fabrication process in that engine parts, seats, wheels and bodywork need to be made and gathered together, along with the workers and conveyor assembly line. Once the components are collected, the growth stage can begin, where cars are 'grown', refined and finally come off from the end of the production line (**). Cars would then be painted, polished and finished for shipping *directly* to the users. Alternatively, rough car shells *plus* a 'finishing kit' (polish, go-faster stripes, extra spot lamps, etc.**) could be shipped (i.e. *indirectly*) to a dealer network. In this case, the intermediate stage customizes and completes the car to the needs of the final user.

By analogy, the tissue engineering system might aim to make a fully finished, customized tissue graft in the bioreactor. Alternatively, the strategy might be to use the bioreactor to make a rough template in which the cells and matrix would mature and remodel after it being implanted to the patient.

Readers may now be feeling a little uncomfortable with this analogy. It is, in fact, not such a good match for the process we envisage, in particular at the two points marked (**). Specifically, cars do not themselves 'grow', of course. The inanimate object increases in size and complexity as parts are assembled by the workers. In addition, the 'finishing kit' supplied to the dealers might be a cute idea, but it

Text Box 8.3 'Bioreactor' caveats – the differences between bioreactor morphs

1. First of all, within the area of tissue engineering, many workers refer to bioreactors in the context of cell expansion systems. In practical terms, these are designed and operated quite differently from 3D tissue-generating bioreactors. Although cells in a natural repair site *appear* to do all things at once, (i) this is, in fact, an illusion, and (ii) 'control' is the real issue anyway. In fact, most cell types are hard enough to control when they are doing just one thing at a time (e.g. dividing or fabricating new tissue. When we aim to prepare as many cells as possible (for cell seeding or injectable cell therapies) the number one target is to optimize *proliferation* in a cell expansion bioreactor. Clearly, then, some of the points in this chapter do not apply to cell expansion bioreactors, where the number one aim is to persuade cells to fabricate as much 3D material as possible. Simplification is essential, so it is best that we concentrate on producing *either*\ cell expansion *or* matrix production systems. Indeed, it is a brave tissue engineer who aims to do both in a single system with our present knowledge base.
2. Much of the discussion here relates to *matrix-rich* tissue engineering, as opposed to *cell-rich* (such as engineering organs like liver or kidney, made up largely of dense cell aggregates). Again, these have been discussed elsewhere and raise somewhat different problems. Whatever the 3D volume available in your particular construct, it will be filled with both (a) living (cells) and (b) non-living elements (extra-cell material, hard or soft, and water) in some tissue-specific ratio. Where (a) above becomes a greater and greater percentage of total volume, energy/nutrient consumption rises dramatically. Where (b) increases, consumption falls in proportion (subject to metabolic activity). This simple balance dominates the difference between cell-and matrix-rich tissue engineering bioreactors.
3. Among the more traditional biochemical engineering types of bioreactor there are a bewildering range of approaches, applications and technologies designed to provide efficient, predictable operation over long periods. For example, there are:
   (a) mammalian cell bioreactors (sometimes transformed cells for reproducibility and enhanced activity);
   (b) plant cell systems;
   (c) bacterial cell systems;
   (d) fungal cell systems.

   There are also simpler, non-cellular bioreactors where whole cells have been replaced by specific biochemical elements required for the reactions carried out. At the other extreme, examples of bioreactor processes are in use with whole (admittedly small) organisms, nematode worms and plants.

Inevitably, there are also many, many technologies developed for achieving the basics such as mixing, separation and recharging of the bioreactor contents. Some examples are given in Chaudhuri & Al-Rubeai (2005).

Reference:

Chaudhuri, J. B. & Al-Rubeai, M. (eds, 2005) *Bioreactors for Tissue Engineering. Principles, Design and Operation*. Springer, Dordrecht, The Netherlands.

hardly parallels the massive tissue-tissue integration and maturation which happens when an implant is sutured into the body. No, this analogy is flawed, as it relies on a human engineering assembly model where the products are non-living systems.

Perhaps a better analogy might be salmon farming. The first stage is the fabrication and collection of the component parts, fish-fry, food, hormone pellets, net pens, circulation pumps and salinity meters. We might envisage an assembly stage where the fish pens are towed out into the bay, the necessary pumps are bolted into position (**) and the cages filled with fish, etc., ready to farm. Then the fish farmer has the choice of whether to take out lots of small salmon for sale to wholesalers, for fattening and packaging for supermarkets, or to grow them to full size for direct supply to the restaurant table and customer.

The flaw in this analogy is a little harder to see, but a closer look shows that the assembly stage process is, in fact, *not* designed to assemble fish, but

the fish farm itself. In fact, the part of this process we control and engineer is the assembly of pens and monitoring equipment, etc. The salmon largely assemble themselves, providing they are housed well out in the bay. So again, this is *not* a great analogy for a controlled process to generate fish. In this case, the bioreactor may more accurately be more identified as the bay where the fish pens are anchored.

The fact that we are having trouble generating good parallels with familiar engineering or farming processes may be an indication that we are missing an important element of what we expect to happen as a tissue is engineered. At the engineering-fabrication end of this spectrum (car assembly), the analogies are clearly rather thin and flawed. A possible mismatch here is that in human fabrication processes, we almost always add new materials or parts *to the surfaces* of a growing structure. We pretty well *never* design assembly processes where the new parts are inserted *into* the existing structures which were made at earlier stages of the process. This is so obvious that it is easy to miss, because it would be too disruptive, in the human assembly world, even to attempt to disturb the 'inside-out' sequence which we might call layer-by-layer or appositional assembly. After all, we (the human producers of the structure) are on the outside and we would like to stay that way after assembly is completed (a rare exception might have occurred in the final assembly stages of the Egyptian pyramids: this is called entombment).

Some readers will be thinking that large buildings are an example of humans constructing from the inside, but this is to miss the point, The 'outer' fabricated surface would extend to the inside of hollow structures. For example, plasterers come in sequence to put a series of smooth layers over the initial bricklayer's work. In turn, these are followed by the painters and decorators, who add ever more cosmetic 'final' layers. The actual building really only gets bigger when more masonry is added to the outer edges of what is already present. No one would suggest inserting an extra row of bricks every metre up the height of an *existing* outer wall to make the building taller.

Not least, assembling structures in this way would mean that all of the previous parts of the structure, laid down at earlier times, would be spatially disturbed, compressed, stretched or distorted, so they would themselves need to be modified continuously during the growth (see Chapter 7). But this is precisely what happens when cells fabricate (assemble) soft tissues during bio-growth. That is, while new parts of the tissue bulk are being added in one area, other areas are being 'remodelled' to accommodate the resulting shape-space changes. A key difference from the human standpoint is that the fabricator-cells *live*, full time, within and *surrounded by* the structures they fabricate. In fact, a much more accurate human-world analogy for tissue-growth would be the rather nebulous process of how we 'grow' our towns and cities. This is not a process dominated by how we construct houses, office blocks, bus stations and football stadia. Rather, it is the process by which we knock down and reshape old, existing districts, roads, business zones, etc. to accommodate the building of new additional city parts (Figure 8.8).

Figure 8.8 Picture of a city from the air (Boston-Cambridge, USA). Imagine that the city fathers want to insert a new Olympic rowing facility on either side of the bridge. This would narrow the river and require the demolition of housing and parts of MIT (red and green circles, respectively). These lost facilities could be moved out by a few kilometres, displacing old factories (yellow circle) to the edge of town (yellow arrow). Meanwhile, the bridge (now too long) is demolished and remodelled to be higher but shorter. This is urban remodelling.

Using the 'urban expansion-redevelopment' analogy, we can suddenly get a clearer view of what makes cell-based tissue fabrication so different from human factories and building sites. Cells are multi-micron-scale factories in their own right, permanently sat *inside* the fabric of their own production. The cells are the 'factories', producing tissue structures inside-out. We can now see why the automotive assembly analogy was flawed. In that case, workers always lay down a steel plate, coat it with protective plastic, then layers of paints and decorative stripes – *strictly in that sequence*. They would never go back at some mid-stage (e.g. after the first coats of paint) to add ribs or fluted shapes into the steel base-structure. The difficulty of imagining how this might be done only illustrates how ingrained our human-scale thinking is (e.g. injecting in more liquid steel in the last example is clearly silly). Equally, revisiting our house-building process, any builders who try to inject extra layers of plaster *behind* the decorative wallpaper would clearly have a short career (not to mention needing possible medical attention).

But if we consider our tissue assembly process to be more like that of city development, things get better. Now, we start to see that the process is really a *combination*, where addition of the car assembly plant and group of workers houses is only a the first part. Expansion goes hand in hand with remodelling of the surrounding buildings (to make space), addition of new road and rail links to bring in parts and workers, telephone lines and offices for the new district government (and tax officials!). In other words, there are *two* sections to what we are aiming to do, and only one of them (construction of new simple structures) can be based on the principles of human engineering and fabrication. The second part of natural growth (progressing all the time, hand-in-hand) is the reshaping of the previously fabricated structures to accommodate the new – i.e. *remodelling*.

This also has implications for our analogies at the tissue-farming end of this spectrum, in particular the difficulties with drawing out good analogies. The fault line here is obvious in retrospect, but its recognition profoundly affects tissue bioreactor logic. It is the problem touched on earlier, of growing fish lips or pumpkin fruit *in isolation*. We never normally even try to farm or culture isolated parts (e.g. single tissues or organs) of our domesticated animal or plant crops. Rumour has it that there were early attempts to genetically engineer chickens with four legs, replacing the much lower meat quality wings. This was never likely to catch on, for reasons of ethics or taste, but it would still not have broken our rule, in that, however many limbs the bird has, whole living chickens would have carried out the tissue fabrication.

Frankly, if there was a realistic possibility of developing processes to successfully farm isolated tissues, we might expect to hear of work on culture processes to grow 100 per cent fillet steaks in huge, sterile vats. Perhaps an early plan might be to grow the most valued Kobe beef steak at anything up to $600 per kg. It is possible to question the validity of this example on the grounds that muscle tissue is complex in structure, must be highly vascular and so is too high a target even for its market value – so perhaps we should instead expect to see a beluga caviar farming bioreactor. Fish eggs, at least are relatively simple in structure and the market value is even greater, currently $7,000 to $10,000 per kg. This is around a third the value of gold (≈$33/gm) but, as a non-durable, consumable item, it could be a marketing dream.

However, the elephant in this room is that farming-type fabrication depends on the inbuilt controls of a whole viable organism. In tissue engineering bioreactor logic, it is rarely argued that we can use the whole organism. Yet, without any real precedent, the tissue engineer's vision of 'growing' functional 3D tissues can start to look like an early alchemist's claims to gold production.

So, to conclude this section, our engineering analogies for tissue fabrication fall down because human fabrication systems are fundamentally different in scale and mechanism to those by which tissues grow naturally. This is aggravated by the realization that hopes of developing successful tissue bioreactors out of biochemical bioreactor (fermentor) technology may not be realistic. Worse still, the widespread assumption that tissue bioreactors

could still work by relying on farming-like process controls looks increasingly rose-tinted. It must at least await serious progress in understanding stem cell and developmental biology, until it reaches the level where single tissues can be grown free of the parent organism.

But before the reader gives up in gloomy despair, it is worth using what we have learned, including the negatives and no-go areas, to help us plot a new 'bioreactor concept'. Key to this is the city planning analogy and the glimpse it provides of a two-part process:

(i) Expansion.
(ii) Remodelling.

In particular, we can see that that our engineering/fabrication skills may be sufficient for assembling simple tissue replicas, but this will not take us further because of our limited understanding of the natural tissue remodelling that needs to happen in parallel with tissue expansion. Therefore, our new concept is a *two*-part process in which the technologies are better fitted to our capabilities:

- The first part involves engineering and assembly of relatively simple tissue replicas or templates.
- The second part would be based on bioreactor-based growth processes, where this template is remodelled, expanded (grown) and provided with bio-complexity by its resident cells.

The problem now is in coming to terms with the concept that we are now *not* dealing with either cultivation or engineering, but *both*. Engineer the simple bulk, then cultivate this such that resident cells complete the second, growth and remodelling process.

Shock, horror, gasp – it's a *sequence*, not a choice.

8.3 Current strategies for tissue bioreactor process control: views of Christmas past and present

This is the point where we examine the stages reached by tissue bioreactor engineering at the present time, irrespective of the logic-analysis in previous sections. At its base, we are focused towards developing systems, chambers and associated processes which will successfully maintain '3D tissue cultures' for extended periods. The purpose of this extended maintenance is to persuade the 3D template or constructs both to mature in composition and architecture, and to grow in functionality, including size, strength or biofunction. For matrix-rich tissue applications, this most commonly requires the accumulation of large amounts of dense, organized ECM (extracellular matrix). For cell-rich tissues (typically organ engineering), there is a much reduced drive for mechanical support and a dense ECM material, but a more pressing need for differentiated cell sheets, blocks or tubes. These would commonly need an appropriate 3D organisation to produce, for example, ducts, tubules, filtration surfaces or vascular integration.

Despite the apparent diversity when viewed in terms of the target tissues, many tissue bioreactors and the associated systems developed so far have much in common, since their resident construct cells must be:

(a) kept alive and highly productive. This involves supplying all the raw materials they need, plus oxygen, and removal of wastes at (i) appropriately rapid rates, and (ii) throughout the 3D volume of the construct, minimizing gradients, except where such gradients are functionally useful (later). '***Construct Perfusion***'
(b) kept active, i.e. doing or making what we need them to do or/produce! This involves '***Cell-Control***'.
(c) kept free of infection from any form of exogenous micro-organism (i.e. sterile, despite all the other comings and goings of nutrient media, measuring probes and additional components). ***'Sterility'***.

Many other (perhaps less fundamental) demands have been added in some cases, including maintenance/direction/reprogramming of cell phenotype, maintenance of stem cell de-differentiation, and generation of cell stratification or (vascular)

micro-tube formation. However, satisfying these three general backbone requirements encompasses most current tissue bioreactor targets.

8.3.1 Bioreactor enabling factors

Construct perfusion

Control or optimization of nutrient and waste mass transport to/from suspended cells has been the central success of conventional fermentor-type bioreactor engineering. It has been one of the central targets to date, with the aim of predicting and then regulating overall bioreactor performance. It is, perhaps, possible to get an unclouded glimpse of how early we are on the *tissue* bioreactor learning curve by examining two of the more basic assumptions for their level of wobbliness:

1. Control of deep cell perfusion is a dangerously low target if we are serious about producing functional 3D tissue architecture. Functional perfusion is only likely to be an enabling factor, either preventing cell death or switching on (or at best speeding up) tissue production. While this is an important basic, as we have seen before, the *speed* of matrix production alone contributes very little to control of 3D spatial organisation.
2. So far, most attention has been on monitoring and controlling nutrient, waste or oxygen levels in the *external medium* in which the construct is bathed, largely because it is technically a simple matter to measure the external fluid. However, recent systems in which micro-monitor probes have been used to measure real-time levels at fixed depths in the tissue constructs have highlighted how dangerously over-simple this approach can be. As already discussed, resident cell *consumption*, rather than diffusion, is frequently the determining factor, but changes in location and activity of cell clusters deep within 3D constructs are not yet predictable. Added to the poor predictability of diffusion properties in the different planes of anisotropic tissues, it become clear that we cannot realistically hope to exert meaningful control over deep-cell perfusion when we only monitor the external culture medium. Indeed, it is worse, of course, as it leads us to imagine that all is well below the surface, so there is no need for further work.

As we have discussed previously, the aim of controlling 3D architecture *or* composition of deep tissue cells or matrix-zones, based on information taken from the construct surface or culture fluid, is either a seriously modest strategy or a massively optimistic target. In biological circles, it is generally assumed that diffusion path length (surface to core) and material density are the factors that govern where and when damaging gradients and deficiencies occur.

However, it is commonly the high density of active cells (in pockets or multi-layers), rather than simple diffusion, which is the determining factor. In other words, it is cell consumption of nutrient/oxygen and production of wastes (discussed in Chapters 3 and 4) which dominates the formation of tissue gradients (actually, extracellular matrix is surprisingly nutrient-permeable). For example, it will be common for the surface one or two hundred microns of cells to be active enough to deplete the lower cell layers much earlier than would be expected by diffusion alone.

Clearly, monitoring the culture medium alone can presently give little indication and very little measurement of such effects. Until our understanding of the dynamics of 3D tissue structure and localized cell consumption are improved, it will be necessary to directly monitor deeper 3D construct layers.

Aside from *measuring* these gradients, current efforts at improving deep perfusion (reducing such gradients) are focused on biomimetic approaches such as incorporation of μ-channelling or blood capillary mimics, with or without cyclic mechanical loading to drive fluid movements. However, as we have learned from past TE strategies, such approaches remain inefficient, random stabs at the problem if they are not closely coupled with quantitative monitoring of actual deep perfusion. This means that we should add to our list of **good TE practice** the need to ensure that analysis of mass transport within the 3D constructs is an integral

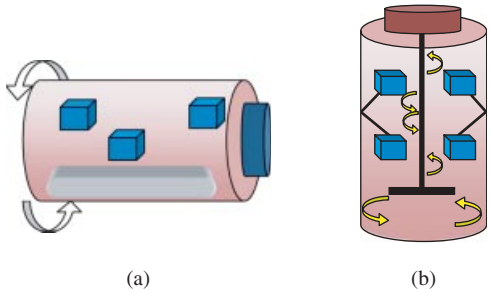


Figure 8.9 Diagrams showing the general principles of (a) roller bottle and (b) spinner flask culture systems. Constructs are suspended in media within the cylindrical bottles on a controlled speed roller mixer. Once they are rotating, an internal ridge helps generate fluid motion, which keeps the constructs moving and suspended. Spinner flasks (b) have an internal free-spinning magnetic bar, turned by a magnetic stirrer under the flask. This generates a gentle (sub-vortex) rotation of the media, around the constructs, held static within this flow on rods.

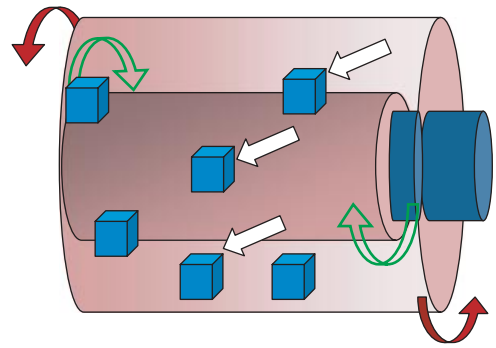


Figure 8.10 Rotating wall bioreactors. Highly simplified diagram illustrating the principle of action. 3D constructs (white arrows) under culture are maintained 'floating' and relatively statically in culture medium by the independent rotation of the inner (green arrows) and outer (red arrows) chamber walls. For a commercially available rotating wall bioreactor, see Figure 8.13.

part of biomaterials or cell biology initiatives to control perfusion. This is likely to become in future, one of the basic norms for 3D-bioreactor targets.

Modern works reviewing tissue bioreactor technology are available, describing recent approaches to the 'construct perfusion' question (see reading list). The earliest of these systems used existing cell culture technologies, which basically agitated or circulated the culture medium crudely around the growing constructs (Figure 8.9). The two basic forms for this were spinner flask and roller bottle cultures. The problem here is that mammalian cells are easily killed or damaged by fluid-shear. Unfortunately, these systems generate relatively high and, more importantly, uncontrolled fluid shear levels, especially around corners and angles of 3D constructs, resulting in local necrosis.

Other forms of fluid exchange can be used. These resemble examples found in conventional suspension bioreactors, where media is exchanged gradually and mixed at the same time by low flow pumps. However, mixing is most critical for the fluid layer directly adjacent to the 3D construct surface, where nutrient depletion and waste accumulation is most pronounced. This layer governs diffusion gradient formation into the construct but, in many cell-seeded constructs, it also tends to impact on large numbers of cells. Consequently, attempts to stir this layer must be highly controlled if damaging fluid shear is to be avoided.

This problem was effectively solved by the development of rotating wall bioreactors (Figure 8.10). These comprise a culture fluid-filled chamber formed between two independently rotating walls. By setting the device walls to rotate differentially at suitable rates, adapted to the construct characteristics, it is possible to generate controlled gentle fluid movements, which keep the constructs suspended at the same height as the construct apparently falls in the opposite direction. In effect, the rate the construct falls is offset by the rotation, such that it maintains its relative position. This also has some appearances of 'culturing under microgravity' (though the accuracy of this idea is contested, it is less important in the present context). The key point here is that the damaging concentration gradients at the construct surfaces can be decreased without generating lethal shear. The result is that cell growth and activity rates in these chambers are widely reported to be excellent.

As we shall see later, other biomimetic approaches to control are now under development, such as the

introduction of perfusion μ-channels and forced interstitial fluid movement. However, these involve tackling the problem at a new (less basic) level, where the construct architecture is designed and prefabricated as part of the process package rather than aiming to produce generic bioreactors that will grow a range of ill-defined tissue 'lumps'. A feature of these perfusion solutions is that they make the bioreactor more complex (within limits) but do not tackle the question of mechano-regulation of key cells within the constructs.

Attempts to control any given mechanical micro-environment on the 3D tissue cells have been reported, and this forms the subject of a later section. The key point here is that this normally involves some form of physical contact – clamping or restraint of the constructs–which inevitably increases the design complexity. Magnetically driven construct loading systems only partly dodge this 'direct contact' point, and they certainly introduce many new variables, such as distance from the magnetic source.

Interestingly, in the case of rotating wall bioreactors, direct physical contact with constructs would be pretty well incompatible with their operation, in effect defining the limits of the use of such bioreactors. But these, in any event, may be better described as minimal-mechanics (rather than microgravity) bioreactors – and this, paradoxically, is not presently a common design target.

Sterility (and scale-up)

Although sterility is included in our listing (albeit under 'enabling factors'), this is essentially a technical driver rather than a concept of tissue engineering (Text Box 8.4). Indeed, many of the basic requirements and solutions are pretty well known in advance. Consequently, despite the critical importance of this aspect and the time commonly expended on its design, it is already well understood from other, traditional disciplines and generally does not need special tissue engineering attention.

However, one key enabling factor has sterile operation at its core, and this is the topic of scale-up. The importance of scale-up is not so much that it is special to tissue engineering – more that tissue engineering is especially susceptible to its application (or lack of). The cross-disciplinary nature of tissue engineering makes its translation to practical applications (including the development of bioreactors) particularly vulnerable to late or poor scale-up design. Since culture systems are initially developed in biological or academic bioengineering labs, it has proved all too possible for them to be developed (too) far down the translation path, to industry or clinic, at the scale of a *cottage industry*.

Text Box 8.4 Sterile and aseptic

Sterile is a very specific term in cell biology. Its absolute significance is worth appreciating in full, particularly to those coming from outside biology. This is simple when we understand how different it is from the term *aseptic*. When we discuss sterility in microbiology, be certain that it means the *absolute* absence of living micro-organisms (i.e. bacteria, fungi, (or their microspores), mycoplasma, viruses or any other non-mammalian cell capable of division). If *any* contamination occurs in culture systems (including bioreactors), the contaminating organisms will eventually overgrow and take over the culture we want to survive, simply because they divide quicker and kill off mammalian cells. The key here is that there are *no* half measures: one bacterium or yeast effectively cancels 'sterility'.

Clearly, non-biologists may have more direct, personal experience of infections, and in such cases then maintaining aseptic conditions are enough. This means that infections are OK as long as they do not overwhelm the *organism*. This last word contains the key factor. The human bioreactor (i.e. your body) has an immune system – the tissue bioreactor does not. As immuno-competent organisms, we can carry substantial loads of 'exogenous bugs' with no bad effect. However, our cultures cannot fight even a single infective bug. Therefore, 'sterile' is the term needed here, *and it is an absolute*.

In other words, the original manual culture process developed in the research lab just grew larger.

'Growing larger', as a cottage industry production, is not the same as undergoing the translation into a scaled up *production* process. This has, in the past, become a fatal problem (notably for skin implant applications reaching the clinic) because regulatory authorities demand that 'the production process' must be rigidly constant once products go for testing. Where implants have gone to trial based on labour-intensive lab scale processing, it is that cottage production process (and *only* that) which the regulators have approved. Once this position is reached, the process cannot easily be redesigned or streamlined for scale-up without entirely re-starting the clinical testing, at major cost. This has resulted in making it near-impossible to generate economies of scale, as the lab scale bio-processing is fossilized into the system.

The ever-present 'sterility-driver' effectively represents a special, and particularly high-profile, aspect of this issue. Indeed, the sterility question can be particularly useful as a warning sign for the wider question of scale-up. Once the question of how to maintain sterility raises its head for any particular bioreactor design, it is probably time to start looking for collaborators in the field of production scale bio-processing. So, we can now identify a new tissue engineering rule for good practice. This proposes that as soon as a bioreactor process becomes interesting in the biologist's lab, and needs special sterilization treatments, it should also attract the involvement of engineering colleagues in order to introduce good scalable concepts.

8.3.2 Cell and architecture control

Control of cell function and tissue architecture during 3D bioreactor operation is the central *active* aim of any bioreactor process (perfusion and sterility being enabling functions). The most important modes of direct cell control tend to fall into two areas, based on the following:

(i) Administration of cell-regulating bio-molecules such as growth factors, hormones, cytokines and gene sequences. Not surprisingly, these tend to be the focus of the cell and molecular biology tribes of tissue engineering.
(ii) Controls which use mechano-regulation. These tend to be more in the domain of the engineering and bio-engineering tribes.

Clearly, for integrated cell control, these groups will eventually need to work in closer conjunction.

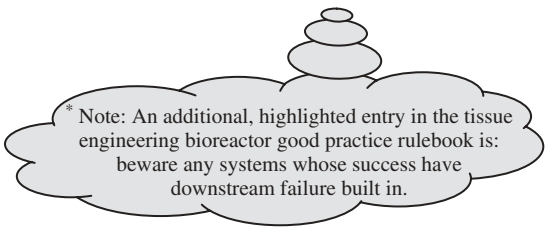
Control using bio-molecular factors

Concepts and strategies for regulation of cell and culture activities by delivering growth factors, nucleotide sequences and other bioactive agents are essentially the same as those applied in general cell and molecular biotechnology. The selected growth factors, antibodies, gene transfection or antisense sequences (for example) are provided, at suitable times, to elicit cell responses previously described most commonly in 2D systems. Using combinations of these, the intended options available are almost endless.

The limiting factors here are practicality (bio-reagent cost/availability and regulatory hurdles) plus knowledge of what they really do, and *how they do it*. Strategies for bio-molecular regulation, therefore swiftly leave the scope of tissue engineering bioreactors, except in one area – *delivery*. Delivery of such molecules to deeper (3D core) parts of our constructs may well be seriously affected by the construct properties, especially the structure of any ECM or support material. This comes about for one quite simple reason: the bio-molecular factors in question range from fairly large to absolutely huge.

Even smaller growth factors or hormones would be in the range of 8–30 KDa in molecular weight, or around 40–160 times larger than glucose. Larger factors and nucleotide sequences would be many times this, making their passage slow at best across even modest path-lengths of dense materials (see Chapter 5, Figure 5.3). Consequently, where such factors are delivered simply, via the culture medium (the most common approach) the material properties of the construct *at the time of delivery* (and not the bio-activities of the factor) will dominate where and how fast they work.

What is more, this confounding factor will change dramatically with increasing culture period in many constructs. The common micro-porous polymer scaffolds will allow relatively rapid and directionally homogeneous diffusion to deeper zones in the early stages, where cell and ECM densities are low. However, with increasing time in culture, this will change in a manner which is both spatio-temporally complex and unpredictable. Worryingly, it will be most pronounced in the most *successful** construct



and bioreactor systems. In other words, there are time-dependent consequences of the cell culture process itself on the rate and directions of mass transport in constructs, which (a) cannot be ignored past a few days of culture, and (b) will be *more severe* and have earlier onset for large proteins, including growth factors. This is why the phrase 'at the time of delivery' is highlighted above.

Immediately after first assembly, constructs are likely to be either micro-porous (>50 μm diameter) or nano-porous in their basic architectures (the latter often being the natural fibrous protein-based scaffolds, e.g. collagen and fibrin). Also, they may vary from highly anisotropic (typically fibrous) to largely random-pore in structure. These extremes will dictate the long-path diffusion rates and (critically) any favoured direction for protein movement when the molecular diameter is a limiting factor. Each 'scaffold' material will have specific properties in this area, and these are usually well documented in the literature. However, even where diffusion rates are rapid and multi-directional *in the early stages* (for example, through large pore isotropic materials), successful deposition of anisotropic ECM and dense cell layers must lead to slower and less predictable directions of macromolecular movement. Thus, long and successful bioreactor operation produces greater unpredictability.

In particular, this unpredictability will affect where and when bio-molecular agents act in the construct. Importantly, this represents a loss of *control*, not loss of action of these agents, as they will still affect the cells they do contact. Where such factors are delivered by addition to the external culture medium (the simplest and most common means), they will increasingly act on the surface cells of the construct and less on core or deeper layers. Where the construct surface is uneven, such as the presence of an incomplete cell layer or physical defects, clefts or channels, there will be zones that allow faster local access.

Experienced construct fabricators will instantly recognize that variations like these in the surface structure are the norm, rather than the exception. They vary between different regions on the same surface, between constructs and, most particularly, between different surfaces of the same construct, commonly as a result of support material fabrication. Such local zoning effects *could* be used to positive effect to generate tissue-like local structure; after all, native tissues are almost never symmetric and homogeneous. However, they are presently uncontrolled – and even unrecognized – variables, and so they further exaggerate the fall-off of process regulation. When the aim is to develop a bioprocess system for controlled fabrication, any strategy resulting in *loss of control* deserves close scrutiny (See tip bubble above, and then Chapter 5).

Control by mechanical conditioning

In recent years, the idea that tissue bioreactors (at least for the connective tissues) should incorporate mechanical cues has become increasingly familiar. Clearly, such a critical, yet imprecise, term as 'mechanical conditioning' deserves a detailed analysis in our understanding of route maps and strategies. Understanding the impact of external mechanical loading on the growth and function of mechanical (i.e. connective) tissues is both a basic need and a characteristic of tissue engineering bioreactors. For now, though, we shall confine the discussion to an analysis of the current forms of 'mechano-conditioning' bioreactors.

For the biological scientists it is important to emphasize that the basic, three-way divide of applied mechanical forces is between tensile, compressive

Text Box 8.5 Applying the force is one thing . . .

Although it is not difficult to *apply* simple forces (tension, compression or shear, in one or more axes) to the target material or construct, what happens to them as they pass through the material is a completely different matter. The more deformable the material of the tissue construct, the more that our 'clean', definable *applied load* is converted into 'other forms' of local forces. We can call these the μ-load patterns, where tissue mechanics gives way to cytomechanics.

Also, in softer materials, more of these complex loads will be translated into deformation of the resident cells. In other words, a uniaxial tensile load applied to a soft, extensible material will generate complex mixtures of compression, shear and tension at different points, even though the dominant overall load is tensile. What is more, the effect of *scale hierarchy* comes in here and these patterns of load are changed dramatically as we slip down a scale to the meso-scale of our cells. External loads applied through stiff materials are transmitted to cells in quite different ways, commonly with much less overall cell deformation, so there is little direct mechano-stimulation.

In other words, as discussed before, cells adherent to stiff substrates are more stress-shielded. The good news is that all of these effects have been understood since Newton's time. They are predictable and calculable – *if* we choose to predict and calculate them!

and shear forces. This allows us to divide tissue bioreactor types along the same basic lines, based on the *principal* class of loading which it is intended to apply (Text Box 8.5). For example, cartilage tissue engineers will generally design systems to apply principally compressive loads for bulk cartilage function with, in some more ambitious cases, shear-loads where the surface is considered. Vascular tissue engineers concentrate on fluid-shear forces for the inner lumen and (pulsed/cyclic) tensile loads on the outer wall. Tendon and ligament engineers tend to concentrate on uniaxial tensile loading. These are based on fairly simple concepts linked to the native properties of the *gross*** target tissue function, reaches *at maturity*.[19]

Examples of mechano-bioreactors are now fairly common and diverse. Figure 8.11 shows two basic types: the pulsing-flow format and one applying uniaxial tensile loads (either static or cyclical). Flow-type culture systems tend to focus on the production of hollow, tube-like tissues such as blood vessels, gut or uro-genital tract tissues. Compressive loading systems, for example in cartilage constructs, are commonly based on commercially available compression test rigs (e.g. Instron-type), applying cyclic loads directly through a conventional incubator wall on to cell-biomaterial constructs in modified culture dishes (Figure 8.12). There is a huge range of possible variants for the means and pattern by which external loads can be applied through even these relatively simple systems. However, it is increasingly clear that next-generation approaches will benefit more from improved concepts of exactly what applied loads are doing at the *cell*** level and in *early*** developmental stage tissues.

The means by which motor power is applied to constructs and their resident cells has involved numerous mechanical loading regimes. These systems have generated much imagination, at least among the biological community. They are perhaps less interesting to the engineering community, where such choices are more everyday-quantitative than imaginative. The most common examples (though not in any order) would be:

- fluid flow (e.g. liquid driven from a peristaltic or syringe pump);
- gas pressure;
- stepper motor linked to screw-driven, lever or cam actuators;
- permanent-magnet impellers.

Each of these can be conveniently controlled (greater or lesser precision) via simple computer

[19]Note: Compare the ** paired terms above to derive our next bioreactor concept-rule, which is that, 'We need to look in the appropriate places when we look to identify mechanical cues directed at the control of *cell* function in *early* stage constructs'.

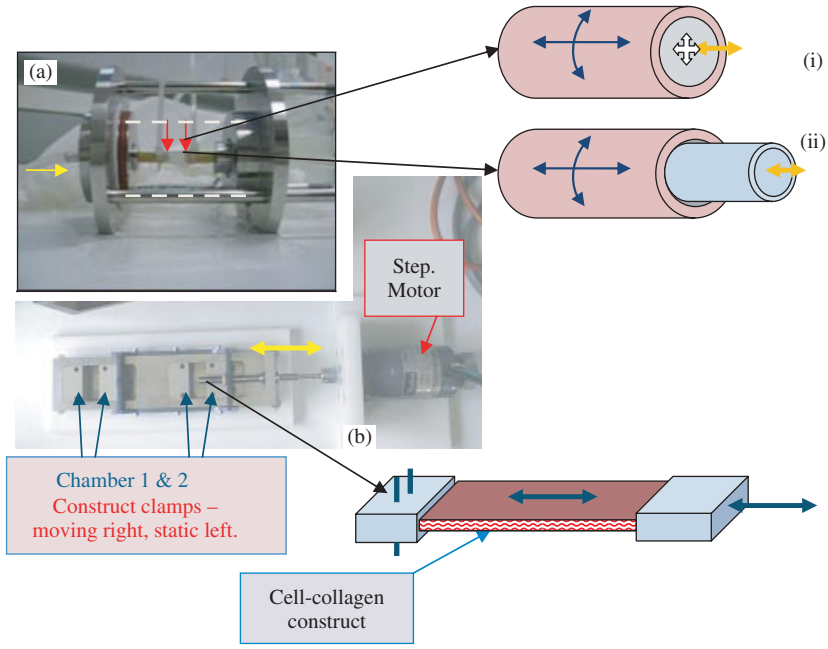


Figure 8.11 (a) Pulsing flow bioreactor comprising a flexible tube construct (red arrows) through which a heavily pulsed flow is driven (yellow arrow). The culture chamber surrounds the outer surface of the construct (glass chamber, white dotted lines, removed here). Set-up diagrams: (i) has the cylindrical construct *only* into the flow line, for lumen shear *and* wall tension (blue arrows); (ii) a flexible silicon lining inside the construct gives wall tension without shear. (b) Stepper motor (labelled 'Step. Motor') and screw-drive provide computer-controlled uniaxial cyclical tension (yellow arrow) onto constructs clamped within two tandem culture chambers. Set-up diagram shows one chamber with (pink) biomaterial-construct, clamped and anchored at one end and load to the opposite clamp.

(a) (b)

Figure 8.12 Instron-based compression bioreactor for 'cartilage' culture. (a) General view showing the external 'Instron' computer-controlled loading frame operating through the incubator wall. (b) Internal detail of the incubator, showing the fittings to load the constructs within a multi-well culture plate.

systems to deliver most patterns of cyclic, incremental or static loading. As a general rule, those in which the motive power is applied most directly (e.g. motor through screw) allow the greatest precision. Non-contact magnetic drives, for example, are exquistily sensitive to the distance betweeen magnet and impeller (variable and difficult to control). Similarly, those where the applied loads are simplest (e.g. unaxial cylic tension) provide the most predictable cell-tisue loading patterns. However, these are made much more complex as they pass through the support materials. In contrast, radial pulse-cyclic loading, generated by peristaltic flow (Figure 8.11a) applied to soft materials, *starts* as dynamic and multi-axial even before it is made *really complex* by the radial, soft architecture of the tube that it acts on.

One of the most easily overlooked motor sources is that of the resident (adherent) cells themselves. Almost all living, adherent cells generate small contractile forces on their substrate which increases over hours after attachment. The fact that this is always present makes it an important factor. It is also important because cells seem to use externally induced alterations to this cell-substrate tension to monitor their mechanical surroundings (Chapter 7). However, in quantitative terms, the forces produced are small and relatively static. They usually have significant affects only on very soft, compliant substrate materials such as weak protein gels or synthetic hydrogels, and then only when cells can *attach* to the gel fibre network.

The concept that the growth of (connective) tissue constructs in culture 'can be controlled and enhanced by the external loads' is, therefore, now well established. This conflicts somewhat with earlier suggestions that micro-gravity culture favours tissue formation in some cases. Micro-gravity, in fact, represents the minimal possible theoretical level of mechanical loading, and it is as far from mimetic, at least of earthbound mechano-biology, as it seems possible to get.

This brings us to an interesting point of logical asymmetry. While it is clear that perfusion-led bioreactor design, such as rotating wall systems

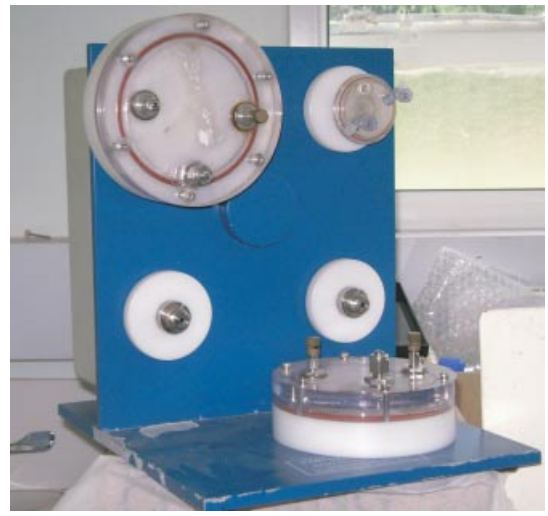


Figure 8.13 NASA-inspired rotating wall bioreactor, said to mimic micro-gravity culture conditions. Constructs continuously 'fall' within slowly rotating culture chambers (one of four) mounted vertically on the blue base-plate.

(Figure 8.13), can make mechanical loading more difficult to engineer, the reverse is true for biomimetic mechano-bioreactors. Where the applied load is designed to generate physiological deformation (i.e. strain) in a repetitive (i.e. cyclical) pattern, these movements can be extremely effective in promoting directional movement of fluids within the substance of 3D constructs. Such *interstitial fluid* movement can be critical in breaking down diffusion and consumption gradients. Consequently, by coupling biomimetic loading regimes with biomimetic 'scaffold' anisotropy, it becomes possible to promote directional fluid movements, so improving deep zone perfusion while delivering cell-organizing mechanical loads. In this way, mechano-bioreactors can become self-circulating and self-perfusing.

To conclude this section, it is important to recap on a number of basic lessons from the development of tissue bioreactor technology (Text Box 8.6). It has clearly been common for biological specialists entering the bioreactor field to simplify the force patterns and perfusion controls needed for their

Text Box 8.6 Balloons in plastic mesh versus cells in collagen fibril network

In terms of tensile properties of constructs made from a number of different components, it is important to understand that there is a logical 'pecking order' for their relative contribution to overall mechanical properties. For example, let us consider a series of small balloons trapped inside a plastic fibre mesh. Most of the stiffness and the ultimate strength (to failure) of the *whole bulk* mixed material comes from the fibrous mesh, which is the stiffest, strongest element. If the fibres are more numerous in one plane than another (e.g. perpendicular to applied load in Figure 8.14a), then the construct will have anisotropic tensile properties (i.e. different in different planes, so *not* isotropic).

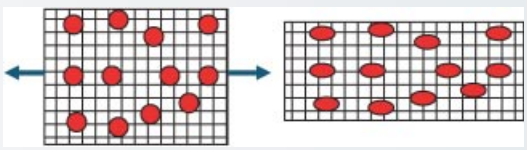


Figure 8.14 (a) Uniaxial tensile load. (b) After loading.

On the other hand, the more balloons we have, the less stiff and easier it is to deform (strain) the material. Since we have only two (active) components, then the greater the proportion of the overall blend is occupied by balloons, the greater its compliance (i.e. strain or deformation produced by a load), as they *must* replace more of the stiffer (less deformable) plastic fibres. Again, if any plane of the construct has more fibres or more balloons than another, then the overall construct will have asymmetrical (anisotropic) mechanical properties. Shifting the balloon : fibre ratio, especially locally, will also tend to change that anisotropy.

In addition, even symmetrically applied external loads can deform the less stiff balloons (cells) asymmetrically, if the gross shape of the construct is asymmetrical (e.g. long and thin). The longer and thinner the construct (called by engineers the 'aspect ratio' – simply length : width), the more the balloons will deform in the long axis, as in Figure 8.14b. As a result, the deformation of soft particles (balloons or cells) will be anisotropic and far more complex than the loads applied. In native connective tissues, collagen fibres are the 'plastic mesh' and cells are the 'balloons'. The strongest, stiffest element is the fibrillar collagen, and groups of cells become the 'weakening' elements. Hence, the greater the cell density in an ECM, the less stiff it will be (in that plane).

Loading analysis of natural (tissue-like) materials, then, should be considered mainly in terms of its collagen fibre content *and its orientation* relative to the applied loads.

tissue applications, but inclusion of engineering specialities seem to help in the long term. There is clearly a set of basic enabling factors for bioreactor operation which must be addressed as housekeeping activities (essential but not enough to achieve our targets). These include sterility, capacity for scale-up and effective deep-zone perfusion. In addition, active factors are needed to control or locally restrict cell activity and architecture. Critically, these active factors *must* be capable of dynamic adaptation to time-dependent changing properties in the constructs.

Nowhere in extreme tissue engineering is the need for engineering and biological cooperation more important than in the hunt for successful tissue bioreactor systems. Separately they are naïve; linkage brings sophistication (Text Box 8.7).

To summarize some of the principles of tissue bioreactor good practice identified so far, we can construct a list:

(i) If it matters, measure it *directly*, at an appropriate scale and time.
(ii) Questions of bioreactor sterility are good markers of the need for scale-up.
(iii) Beware 3D bioreactors with a tendency to generate damaging conditions where they are successful.
(iv) Selection of control cues is scale- and stage-dependent (we work mainly at the 'cell' and 'early').
(v) 3D bioreactors designed by only one tribe are likely to be naïve in one major sector or another.

Text Box 8.7 Some clues and mnemonics for mechano-bioreactor function

(a) In Mechanical Loading and Connective Tissue Engineering: **Newton Rules!**
(b) In *Connective* tissues (almost) everything is connected, so forces *transmit*.
(c) Material randomness/anisotropy governs force transmission.
(d) *Force Vectors* interact with *Material Anisotropies* to produce **change & complexity**.
(e) **OOPS** (out-of-plane stimulation) and stress-shielding are key to cell mechanics.
(f) Bioreactor culture *duration* alters the transmission of both load and growth factors.

Look up Newton's Three Laws of Motion: how do they apply to the diagrams in Figure 8.14 (Text Box 8.6)?

8.4 Extreme tissue engineering solutions to the tissue bioreactor paradox: a view of Christmas future?

At the end of Section 8.2, we left things a on a bit of a cliffhanger. Where should we go next if basic tissue bioreactor strategies have so many flaws? In a nutshell, tissue bioreactor logic sits uncomfortably at the crossroads of two other strategies:

- Conventional engineering-type biochemical bioreactor technology.
- Advanced (whole organism) cultivation-type technologies.

This seems to have led to strategic targets for *tissue* bioreactors which seem unlikely to prove realistic.

So, to pick up the story again, our task from this position must be to plot out fresh forward strategies and identify the log-jam points that they inevitably contain. Options include:

1. Perhaps we can progress with the use of engineering-type fabrication processes by minimizing our dependance on controlled cell activity (i.e. sticking to what we are good at). This would involve applying strictly human-type fabrication processes to assemble simple, bulk material templates of the tissue. Any cell-dependent processing would be used as little and as late as possible. Put another way, this would involve translating how cells fabricate matrix and tissues into human factory-fabrication systems – more like Ford or Sony and less like salmon and pumpkin.
2. A second, almost 'looking-glass' approach exists for those who favour reliance on natural biological processing. This maintains that ways *really can* be found to cultivate tissues in 3D if we work at it. The first exteme version of this route involves actually growing constructs within whole organisms (i.e. in the patient). This is the so-called 'human bioreactor'.
3. The opposite extreme of bio-cultivation strategies proposes that we learn how to recapitulate how tissues developed *in utero*, then translate that into growth *in isolation*. In other words, the target here is to engineer developmental biology and (parts of) foetal growth.

Although the last of these sounds dramatically ambitious, it has its advocates. It also comes with an obvious, but essential, first requirement – a catalogue of very special cells. These cell types and cell stages would be comparable in their programming to the tissue producers in a developing embryo. Hence, the hunt is on to mimic embryonic stem cells, or to reprogram adult-derived cells and to determine the means to control them. The reader will recognize this as the logic-stream which underpins present stem cell, regenerative medicine resarch.

8.4.1 In vivo *versus* in vitro *tissue bioreactors: the new 'nature versus nurture' question?*

In traditional biology and bio-philosophy, there is an interminable argument about the proportion of

the total adult organism function, which comes from inbuilt genetic information, and how much is dictated by the environment. Psychologists have refined this for human consumption into the nurture-nature debate for child development.

We now have a comparable discussion in tissue engineering. In this case, the 'nature' idea suggests that cells with the right programming, and a good 3D template, will grow into a functional tissue in the best and fastest manner, in an *in vivo* implantation site. In this logic, engineering 'control' is minimal and innate biological processing is maximized ('minimal bioreactor' input: Figure 8.15) The idea of directly engineering or fabricating tissues represents the opposite of this, or the 'nurture' track where the environmental factors are controlled and engineered (i.e. 'maximal bioreactor' input: Figure 8.15).

8.4.2 Do we need tissue bioreactors at all?

As we have seen, there is a widely held idea that it will be possible to use the natural growth process and to delegate control to the cells. This draws on the logic of tissue farming and embryo development. In effect, the concept of '*in vivo* bioreactors' suggests that even less basic knowledge or biological control is needed if we can combine innate tissue-building behaviour of our seeded cells with the support provided by the implant site itself. In effect, providing the human recipient (bioreactor) of the engineered implant with three meals a day and a warm room could be a more efficient growth machine than existing costly and imperfect bioreactors.

Indeed, conditions for growth and repair in normal adults are extremely favourable for bulk tissue formation, although, admittedly, this can produce scar tissues and is far less effective in the sick and or aging, who are mostly the patients. In this respect then, *in vivo* bioreactors, where they work, will have attractive growth rates, perfusion and matrix deposition rates. Sadly, where they do not produce suitable tissues (see Section 8.1), there is little to be done, because the tissue 'engineer' can exert little real process control.

Nursery implant sites

The concept of an implant nursery site is a version of the *in vivo* bioreactor, though, in this case the surgical nursery site itself is designed to allow a partially formed tissue template to mature and develop function. This has been suggested and tried, particularly where strong vascular in-growth is important (e.g. in bone). The nursery site can be quite remote and very different to where the implant will ultimately be used. This allows for the selection of sites

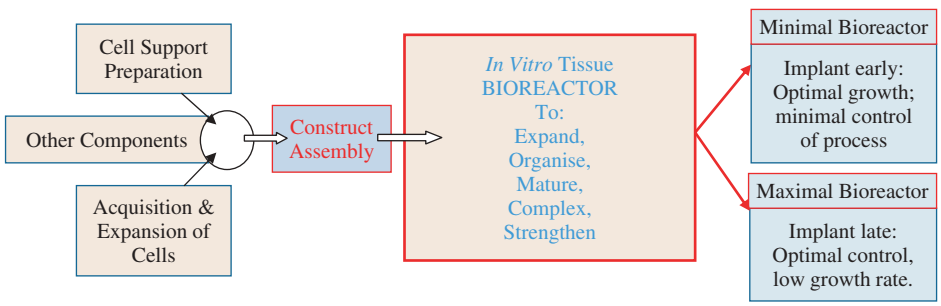


Figure 8.15 Diagram summarizing a standard process sequence assumed to be needed to fabricate tissue constructs. The left-hand cluster of boxes represents the acquisition and preparation of the basic components. These various component elements are then assembled into the initial construct (cells seeded, growth factors/drug depot inserted, etc. – small central box). The main box represents the 3D tissue bioreactor operation stage, which can be brief, allowing minimal cell integration/attachment prior to implantation ('minimal bioreactor' effort) or long, to allow the resident cells to fabricate tissue bulk ('maximal bioreactor' effort). Versions of this can range between maximum and minimum bioreactor effort. Maximal effort involves retaining control of the process conditions, but at high operating cost and slow construct growth rates. Alternatively, tight process control can be given up early in exchange for economy and speed.

which favour good tissue growth with less chance of damage to fragile early tissue templates.

For example, a template construct might first be implanted into a highly vascular tissue bed of choice (e.g. a large skeletal muscle), rather than the ultimate functional site, say in a bone. At a later stage, the robust, mature construct would be moved (with its vascular supply) in a second surgical operation. This option adds something to the speed of construct growth and integration, but these factors must be balanced against the cost, pain and risk of extra surgery on the patient.

The balance between in vivo *and* in vitro *bioreactor (early versus late implantation)*

Aside from the patients' view on extra surgery, the problem with *in vivo* bioreactors is that they largely give away operator control and so become skill-based, one-off surgical events rather than controlled bio-engineering processes (Figure 8.16). There can be little process control and systematic, predictable tailoring to the needs of the patient (age, disease status, gender, etc.). The task of the tissue engineer in such early implant processes is to fabricate initial templates which carry with them inbuilt controls and cues that direct local repair, with minimal scar tissue formation. These need to operate long after the construct is implanted (i.e. prolonged bio-control processes need to be inbuilt during 'construct assembly', to operate long after the implantation, when it is unlikely that external controls can be effectively applied – Figures 8.15 and 8.16). This key tension is illustrated in Figure 8.15.

The early implantation of simple stage constructs has plenty of attractions, but it gives away control to the very vigorous *in vivo* repair processes. Retaining these controls with a prolonged *in vitro* bioreactor process offers the possibility of eventually getting the tissue quality and function that we need. However, the longer the *in vitro* stage, the more technically challenging are the control processes, and the greater the cost, complexity and time taken to achieve any function.

Currently, conventional 3D tissue bioreactors require weeks or months of culture time, and even then construct function is frequently poor or well below that of mature tissue. The compromise is to implant as soon as possible *after* we have cultured a basically functional 3D tissue. This is easy to say, but difficult to achieve. The question, then, is: 'What is the minimal culture period needed to get a useable graft template?' We can be sure that the answer will not be satisfactory until it is reduced to hours, rather than days or months.

The answer to our question here – 'Can we replace *in vitro* bioreactors with early implantation to an *in vivo* bioreactor?' – would seem to be 'no' in most cases so far tried. However, the question itself is almost certainly faulty, as we probably cannot

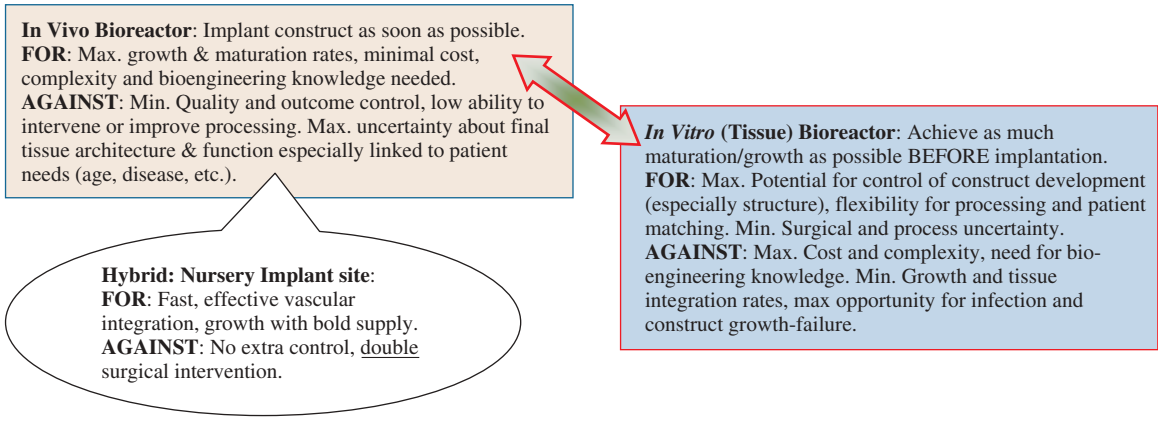


Figure 8.16 *In vitro* versus *in vivo* bioreactors.

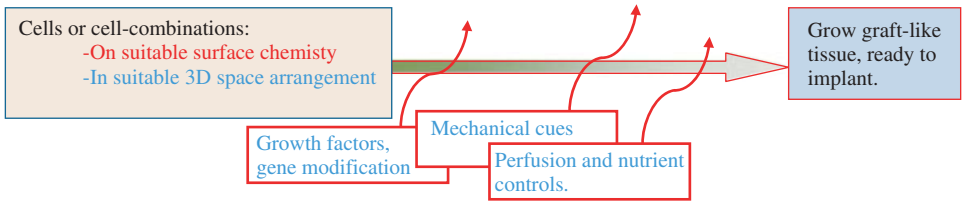


Figure 8.17 Overall summary diagram of bioreactor logic.

eliminate either form of bioreactor completely. In reality, we are discussing the timing where constructs are shifted from one state to the other (i.e. the length of the arrow in Figure 8.16).

This is where the fudge emerges. Each tissue and surgical procedure will require a different degree of pre-implantation 'control'. Where functional architecture can be generated quickly, constructs can be implanted shortly after construct assembly. Where greater levels of biomimetic complexity are needed, the process is forced towards a longer and longer *in vitro* bioreactor stage. In these cases, the technical challenge is substantial, as the cost and failure rates of existing systems will be impossible to carry into routine mass clinical use.

We need radical new concepts to reduce the time-dependency of current bioreactors.

How to maximize the cell-based contribution to tissue production

From the previous section, the question arises of: 'How could we develop and improve the innate ability of cultivated cells to produce the 3D structures we need?' In effect, this asks how far we can go *in practice* using cell-dependent biological cultivation technology. The question falls squarely in the tribal homeland of the cell biologist. It is at the heart of the most familiar of bioreactor philosophies but also the area least likely to give definitive quantifiable answers. It is based firmly on the assumption that, if we acquire just the right cell type or combination and give them cues they can follow, they will 'make' the tissue we want. The trouble is, as with the Hypocrites caveat, we shall never know if it is possible until we have done it.

Figure 8.17 illustrates one way this can be summarized. The current focus is on selection and pre-conditioning of the all-important cells in the construct. As we have considered earlier, this will involve the isolation and purification of cells, with promotion of differentiation towards defined cell types. Triggering cues here, as we have discussed already, may be nutrient, growth factor or mechanical in nature. In previous eras of tissue engineering, the focus has been on finding key growth factors or perfusion levels.

Finally, to return to our analogy of pumpkin cultivation, this strategy is like providing suitable nutrients at one stage for leaf production and then, later, shifting this to another to promote flower formation. After pollination (mechanical stimulation), fruit formation is triggered. The principle of this approach is consistently to use bio-technology stimuli to trigger each successive 'next *but natural*' stage within the bioreactor environment.

After each stage trigger, the natural growth process is left to take care of the complexities of completing the stage detail. We do not need to control the detail of flow shape or pumpkin fruit water content. The process triggers onset and the organism sorts out the detail (Figure 8.18). We shall use this process analogy to link into the next part – 'Process analyses' – in Chapter 9.

8.5 Overall summary – how can bioreactors help us in the future?

The problem and background concepts analyzed in this chapter are probably the trickiest and most intractable that we have to tackle. Not least, this is

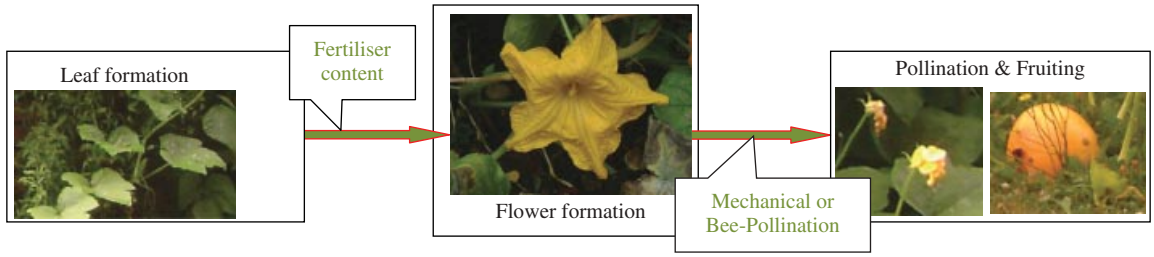


Figure 8.18 Pumpkin bioreactor analogy: bio-artificial triggering of native sequence of cultivation stages. Cultivation conditions are maintained throughout with staged input of triggering cues, provided as the process controls. Between the process control/triggering stages, the biological system (i.e. the pumpkin plant) controls itself and its growth process, with the support of the basal bioreactor environment (culture conditions: perfusion, mixing, temperature etc.).

the focus point at which a number of theories of how tissues can be built meet with the cold water of what is presently practical. In the past, the big question has been 'How can bioreactors help us engineer tissues'? In fact, it is possible that we should flip this question[20] and ask: 'How can the field derive functional tissue bioreactor systems?' as this, in fact, is *the* core problem. In other words, we are not going to be able to engineer tissues until we can conceive logically of *how* to do it. This idea suggests that we may have even further to travel along the learning curve than we had imagined.

The strategy here has been to break the issues down into digestible lumps (questions of dynamics and monitoring are saved for the next chapter). In the first instance, we identified the existence of discomfort among the ranks of at least one of the tissue engineering tribes, with the assumptions of engineering tissues in 3D culture. This we could expand on to identify the presence of two competing but fundamentally different concepts (engineering and cultivation) of how to make 'things'. Both are applied to tissues, yet each has its own enormous logic flaw which would take a great deal of work to get past.

This became very clear when we tried to draw out parallels or analogies of making tissues based on how either (i) engineering or (ii) cultivation technologies work at present. However, the building of analogies did lead us to a new concept, based on how most tissues really do grow. This is the idea that we should compare engineering tissues with how we achieve urban redevelopment, rather than growing or fabricating their components. This comes in a two part sequence; build and remodel.

At present, there are probably three threads of bioreactor thinking, which are freely intermixed:

(a) Minimal *in vitro* bioreactor use; implant cell-seeded support materials to mature/remodel *in vivo*.
(b) Maximize the culture and cell-based bioreactor role (cell-dominant, farming type).
(c) Maximize the engineering and fabrication contribution to the technology (i.e. minimize cell-dependence).

In effect, these three threads curently wrestle with the problem of 'when to tranfer', from (i) *in vitro* to (ii) *in vivo*. The idea is that if we knew where best to put that transition point we could design suitable 3D bioreactro culture systems to service it. **Early implantation** equals simple, inexpensive bioreactors; **late implantation** means we need increasingly complex sytems and processes. However, this point merges with our creeping suspicion that instead of either one or the other approach being better, we might really need *both*, within a *two-stage* process (ie. the 'build and remodel' urban re-development

[20]The origin of some of the simplest yet strongest of insights can lie in an inversion of a basic question, as in: 'Ask not what your country can do for you . . . ' (J.F. Kennedy).

analogy). In fact, the early/late debate may be off-topic and not helping at all.

So, what if tissue bioreactors are best thought of as two-stage processes? Suddenly, there is a glimpse of a rational strategy. The template 'building' function is suited to engineering approaches, while growth and maturation (bio-functional remodelling) is best carried out by cultivated cells. Only then, as a third step, would the construct need to be implanted, for tissue integration. This now gives us two transition points, so – *a sequence* ! It implies the need for triggering changes leading up to each transition, which must be monitored and timed. In other words, we have derived an outline plan for a dynamic *process* to make tissues. This has to be progress over an all-or-nothing structureless tussle between imperfect 'alternatives'.

In the next chapter, we consider more fully the demands and opportunities of the fourth bioreactor dimension: time, sequence and process dynamics.

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