Biodegradable scaffold – also known as 'Builder's Bamboo'. This scaffolding, erected in Shanghai for the repair of low-rise buildings, can be regarded as 'biodegradable' for civil engineering. We expect it to gradually degrade over time through environmental effects. Inset: bamboo scaffold, ready to go.

4 Making Support-Scaffolds Containing Living Cells

Bulk material compositions for holding cells naturally

4.1 Two in one: maintaining a synergy means keeping a good duet together

Those readers with previous experience of tissue engineering might notice something a little out of the ordinary with the scope of this chapter. There is a strong thread in the field which likes to deal with the 3D biomaterials (scaffolds) as one distinct issue. The acquisition, processing and expansion of the various possible cells which are likely to be used is then introduced as a quite separate subject. At first glance, this seems to be a pretty reasonable structure, based on a mix of logic, habit and expedience. After all, these are two very different disciplines. Also, when we are faced with major problems, it can be good to separate the component tasks, often into the main areas of expertise. However, this must still be balanced by the core tissue engineering need for joined-up, collaborative thinking.

As an example, we can be pretty sure that the value of joined-up collaboration is well known to the world's largest shipbuilders, Hyundai Heavy Industries of South Korea. When they start on a new mega-ship design, it is certain that the people

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

running the flow simulation tanks, complex as their task is, are in close contact with their colleagues who design the internal space and accommodation. Furthermore, neither would feel comfortable about spending much time on a design without consulting the marine propulsion (engine) section. After all, no one gets paid until the ship (i.e. *the functional unit*) can carry what the customer wants, where and how he wants it carried. For example, cargos as diverse as 0.5 million boxed Barbie dolls, 3,000 paying passengers, two roads worth of course gravel or a kilometre-long tank of Saudi Arabian crude oil all need to be carried where and when they are required.

Indeed, shipping and tissue engineering could easily share a motto: 'functional carrying devices should be fully adapted to protect and carry their specialist cargo wherever that cargo needs to go – or there will be tears'.

In tissue engineering, two critical groups who commonly collaborate come from very different (disciplinary) tribes. These are the cell biologists and polymer chemists/material scientists. We can just about imagine (Figure 4.1) the level of commercial disaster that would result from the appearance of a gleaming new bulk oil tanker in place of a passenger liner for the return leg of a Caribbean cruise. No matter how carefully the stripy deck-chairs are positioned between the oil discharge pipes and gas vents, 3,000 hungry New York tourists would *not* be amused by the new accommodation as they slip out of the harbour at Antigua.

It is likely that tissue engineering cell-scaffolds can suffer at least the same disastrous level of functional mismatch. Furthermore, the consequences are likely to be equally dire, though we may still have a lot to learn about recognizing and measuring such functional clangers when they occur in tissue engineering. It is not a bad basic lesson, however, based on the ship-design analogy, to keep the key specialist tribes working closely together. As we saw in Chapter 1, maintaining close contact between tissue engineering tribes is critical – so what better way than to merge their most important *shared* contributions into a shared chapter?

4.2 Choosing cells and support-scaffolds is like matching carriers with cargo

After examining how the diversity of disciplines contributing to tissue engineering have shaped the subject (Chapter 1), it may not be a surprise that the tangible effects of that diversity are still emerging. As we have seen, one of the earliest concepts was that the building blocks for the fabricating new tissues would be:

(i) one or more 'suitable' cell types (ideally with stable, tame and reproducible characteristics, matched to the target tissue);
(ii) a 3D porous, cell-support scaffold, 'suitable' in its physical characteristics (mechanical properties, pore size, connectivity and so diffusivity) for cell attachment and synthetic activity.

(a) (b)

Figure 4.1 A blissful outbound voyage though the Caribbean on the newest cruise liner would soon lose its magic if passengers were met by an oil tanker for the return journey. Photo (b) © iStockphoto.com/phlegma.

Instantly it is clear, though, that these two central pillars of the subject are buried deep in very different disciplines – one being cell biology, the other biomaterials science and polymer (including natural protein/polysaccharide) chemistry. In fact, task delegation between the two (cell and materials) tribes could not have been easier. In effect, a common operating pattern of work has developed in much of tissue engineering based on:

- 'We can make some scaffolds, can you find some cells that will live in them?' or
- 'We can generate some really promising cell types. What have you got for us to deliver them in?'

The reader might get the impression that this resembles more of an after-the-event collaboration than an example of ground-up biomimetics.

The luxury of hindsight allows us to question why trial-and-error experimental cycles of different cells in different matrices kept going for as long as it did without a more critical look at its basic logic. There has always been a chance that, one day, it would generate a serendipitous discovery of some special technique for really good 'tissue' growth. In the main, however, we have not been outstandingly lucky in this. It turns out, unfortunately, that it was not such a benign circular scrubble as it seemed; there was a hidden downside.

In fact, keeping the two sides of the 'how do we make a 3D tissue' question separate has encouraged a completely artificial and unhelpful separation into two approaches and philosophies. Part of the tissue engineering community has spent its time asking:

(i) What cells shall we use for 3D culture and how do we get hold of them?

The rest have been concerned with:

(ii) What is a good scaffold material to carry someone's cells and how big do the pores need to be?

As with most imperfect logics, the flaw soon floats to the surface as an even more difficult problem. In this case it immediately creates a third, and unavoidable question:

(iii) How do we get the cells selected by one group of workers *into* the material (fixed in place and living) developed by the other?

In effect, by segregating the two core tasks, we may have made things harder, by expanding the need to *merge* the other two, i.e. creating **the cell-seeding question** (Figure 4.2).

As we shall see later, this is no shrimp of a problem – it is a great white shark of an issue which

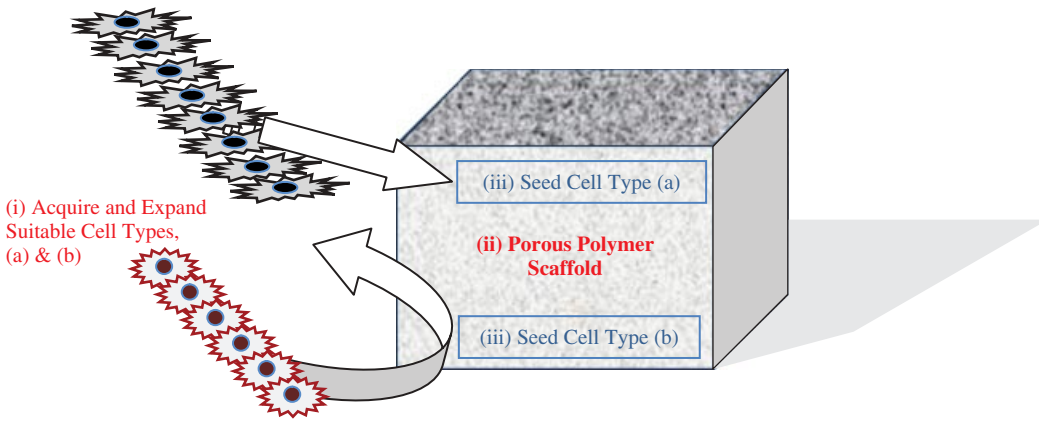


Figure 4.2 The idea could not be simpler or more basic: (i) Collect and expand cells which we hope will synthesize and assemble the tissue fabric. (ii) Fabricate a porous polymer which will temporarily hold these cells in appropriate 3D conformation. While this makes it easy for the two tribes to work on the individual components – it also means we have created a third stage. (iii) Cell seeding.

Text Box 4.1 Exercise 1

Imagine you are trying to find the most bizarre and incongruent mismatch of cells and substrates you can possibly think of, in order to generate models of cancer or diseases.

- Design a research team by linking together three different specialist groups into a collaboration, and justify your selection.
- Then design how you would instruct them to go about hunting for ways to generate the most non-physiological, pathogenic cell and matrix cluster they can manage.
- Now give four examples of how you would measure the construct properties after two weeks in culture, to demonstrate how 'distressed', disrupted and diseased your 'tissue' had become.

Tip: *Imagining you are a member of one of these groups will help you explain your plan.*
[Guideline: approx. 2 pages: 60–90 min.]

surfaces again and again to rip lumps off our best efforts.

Aside from generating an extra question, this artificial division makes it much easier for experts to pre-design the 'solution' around either a promising cell preparation or good-looking scaffolds. Unfortunately, each of these approaches on its own is only likely to generate poor tissue engineering solutions (Exercise 1 in Text Box 4.1). This is the metaphorical equivalent of a bulk gravel carrying ship waiting patiently at Rotterdam's busiest refinery wharf to load up with liquid gas!

What once seemed a prudent and conservative approach can now appear, in retrospect, to be just too low a target to have been realistic. In our shipping analogy, we could imagine trying to salvage the situation by welding and sealing the hatches, bulkheads and drain-channels in our gravel carrier so as to hold in a few cubic meters of liquefied gas. Really, though, it should be clear that we are labouring to fix a problem of our own making. Now hold on to that thought for a while – we shall return to it shortly. This issue forms the core of our first glimpse of extreme tissue engineering for this chapter: the case of '**aiming low and still missing**'.

4.3 How like the 'real thing' must a scaffold be to fool its resident cells?

The 'real thing' here, of course, is the natural mature, healthy tissue in question, with all the complexity implied by that phrase (see Chapter 2). The assumption in this chapter will be that it is, at least in the early stages, both logical and pragmatic to develop cells and their support material as a single, integrated unit. To do this, it is first necessary to understand the basic mechanisms by which our chosen cells will use their 3D surroundings (Chapter 3). The idea is that this will give a good indication of the *essential elements* we need to mimic for this or that cell-tissue-injury system. We might reasonably expect such intellectual tools, or basic knowledge, to enable us to design genuinely functional 3D cell-support systems.

The more astute (or wizened and cynical) readers may detect the faint odour of 'shallow logic' in the last paragraph. Although it is apple-pie-obvious that we should get enough background knowledge in the first place, in biomimetics we have the greatest difficulty predicting when we have 'enough'! A slightly more honest question might be to ask, What are the five or six top features that a 3D support material really *must* have if it is to fool our (*insert name of our cell type*) cells to doing what we need of them? This really is a crucial question, but it is so full of caveats that it merits a full in-depth analysis.

Firstly, the choice of five or six features is pretty arbitrary, though it is, empirically, a useful starting point. The history of the field suggests that, where it is known, the identity (and significance) of a reasonable spread of features can make it is possible to make a half decent stab at a design. Five to six generally does give a reasonable spread! The selection criteria are important, though. These are *must-have* features, necessary to 'fool cells' – the classic core of biomimetics – not features to satisfy

government regulators or university accountants, but must-haves from the viewpoint of our cells. In fact, we are looking for the least we can get away with in terms of bio-compromise. After all, if we do not find simpler compromises but aim *only* to be more and more 'like nature', our solutions become incredibly complex and impractical to produce – in fact they *become* natural systems rather than mimics.

There are informative examples of the 'simplest effective signal' principle in nature, which are both illustrative and useful for calibrating our estimates of how simple 'simple' can really be. Figure 4.3 shows one: the red dot on the lower beak of a humble herring gull. Possibly you have noticed this while you were at the coast; or more probably not. But it has not escaped the bird behaviourists, who concluded that this dot is the target at which gull chicks aim when pecking at the parent's mouth. When the chick pecks at the beak-spot, the parent is stimulated to regurgitate partly digested fish, which is, oddly, appetizing food for the young gulls. For the gull this is a simple but effective information loop. The point is how surprisingly simple this signal is. Indeed, the chick will perform the full pecking ritual at any red spot painted on a piece of wood. The rest of the parent bird is unnecessary, except of course to supply the half-eaten sardine.

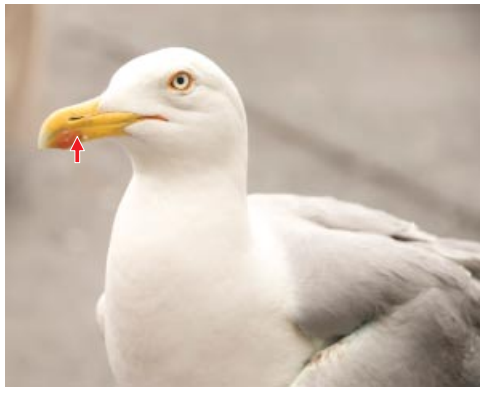


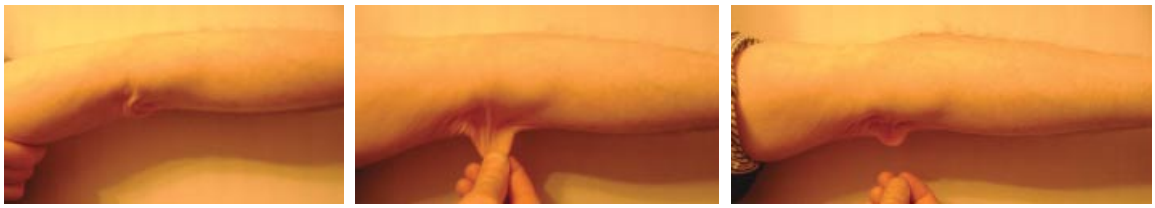
Figure 4.3 The humble herring gull has a red dot on its lower bill (arrowed). This demonstrates what we are aiming at in biomimetics, as the dot is all that is needed to stimulate the chicks to feed from the parent's beak. So strong is this key message that chicks will peck at a white stick if it has a red spot painted at one end!

This example illustrates how we can sometimes fool bio-control systems by input of really easy, simple pieces of information, *once we know what that minimum is*. If we were into gull engineering, we might generate energy from dot-pecking chicks. For tissue engineering, we need to know the minimal cell-cues needed for our 3D material supports.

The problem is the huge breadth and detail that these cues *could* take. We can start with the surface chemistry of our material. This modifies how different proteins from the body fluids bind to the surfaces when they are implanted, and so how cells attach (or don't), because, of course, different cell-types use different attachment proteins to go about their business. Then there is the substrate stiffness, or lack of it. There is much more to this than high and low 'squishy-coefficients'. Cells in natural tissues are adapted to (and so respond to) physiological tissue material surfaces with complex 3D zones and layers. Sometimes these can have continuous stiffness gradients. You can find examples of these for yourself; pinch your skin in a series of close places, running around from the back of your neck to the front of your throat. Alternatively, gently stroke the skin the back of your hand up and down and watch what moves. Now keep stroking and looking, but move around to the palm and see how much less moves!

The possibilities get more daunting still when we realize that these material properties are normally asymmetric and, worse still, they can be dynamic. 'Dynamic' means that properties change with time and even with rate of motion, as the materials move and water is displaced (visco-elastic behaviour). This happens during simple, everyday bending, stretching or compression. Such movements generate many secondary effects on cell physiology, for example enhancing tissue fluid movement/perfusion parallel with (rather than across) anisotropic fibre materials.

You can get an idea of the rate-dependent and dynamic tissue properties by doing the stretching-your-elbow-skin exercise. Figure 4.4 shows how the loose skin over your elbow will stretch and bounce back. This is both age- and direction-dependent. Snap-back becomes faster and greater where we test along a father, son, grandson series. Interestingly, the recoil tends to be complete when you bend



(a) (b) (c)

Figure 4.4 The elbow skin snap-back test. Try it.

and then unbend the elbow, but incomplete if you pull down and release (as shown in Figure 4.4c). This suggests that the direction in which the skin is extended matters, i.e. it is anisotropic.

We can see that factors which mimic *special* features in the cell environment not only offer a rich vein of control mechanisms but also represent small parts of very complex systems. Our extreme tissue engineering task is to identify the critical but minimal components of these complex spatial-mechanical cues which cells can recognize as 'key signals'. In other words, what are the 'herring gull red dot' factors?

In contrast to this biomimetic hunt amongst complex natural signals, cell-support materials (scaffolds) are presently selected and designed on biomaterials grounds. These are, at best, rather simple, identified largely in the search for prosthetic biomaterials. Indeed, the assumption that ultra-simple, even non-mimetic factors can determine cell outcomes seems to be at the core of the tissue engineering dream. The dream suggests that we will persuade cells to recapitulate biological fabrication with simple biomaterials, plus a handful of other cues, including protein growth factors. When it comes to designing the materials, we hope these will provide the special, key cue (gull-factor) deceptions.

As a result, a modest number of options are regularly revisited, occupying a great deal of literature with increments of new cell types on slightly altered surfaces. Some are simple tricks, learned from cells in monolayer culture, such as surface patterning. These can include producing grooves, channels, pits and humps in the cell-support material surface, or occasionally between layers, where we are dealing with the cues deep in the bulk of materials. Indeed, these can be potent cues to guide cell motion and cell shape. They can indirectly alter behaviours such as proliferation, differentiation/gene expression or protein synthesis and export. However, they are manly derived from phenomena in 2D monolayer cultures. In effect, they are highly simplified parodies of natural control systems, based on little knowledge of their operation. Worryingly, these may be our best examples.

Other popular design features concentrate on factors such as material porosity and the degree of pore interconnectivity. This is the inevitable legacy of the need to get cells to migrate deep into many conventional support materials. They are the consequence of the cell-lethal conditions used in polymer and/or 3D scaffold manufacture. *Porosity* also gets far higher up the 'must have' list than it deserves in response to the largely faulty dogma (see below and Chapter 3: Mount Doom) that cells will die in a hideous, hypoxic agony if not adjacent to medium.

Worse still, it is still possible to see key design criteria quoted which include the words '*cheap*', '*generally biocompatible*' or '*approved by government regulatory offices*' (such as the FDA). To have these in the 'top 10 of biomimetics' seems to represent a grand misunderstanding of the high aims of biomimetic engineering. In particular 'cheap' is as appropriate as souring economy aluminium alley for Rolls Royce turbo fan blades.

Based on such analyses, the newcomer could be forgiven for thinking that tissue engineering is an ambitious dream, populated with extremely low targets. For example, when we make a successful off-the-shelf skin graft to treat the most high-burden problems of aging Western societies, its high market value will definitely *not* require that it is made from

cheap, recycled waste products. Similarly, if we are intent on making *absolutely nothing* that could be new, progressive or adventurous, then sticking firmly with previously FDA-approved starting materials is clearly the way to go.

Indeed, as pore sizes evolve to greater than a few cell diameters (i.e. towards being *less* mimetic), we now see that they actually undermine (albeit accidentally) a central requirement – namely, 3D cell growth (see Chapter 3). Other factors become poorly mimetic as a result of such dubious, ingrained priorities, including:

- inappropriate cell clustering;
- sharp and distorted nutrient and gas perfusion gradients; and
- non-physiological access to, and export of, bioactive proteins, especially in terms of *rate and direction*.

More of these later.

In contrast, systematic identification of truly defensible 'simple' cell-cues is still in its infancy. Examples include:

- Fine grooves which stimulate many cells to elongate and align.
- The use of very soft or much stiffer materials to elicit fibroblast quiescence and low motility, as opposed to cell division and migration.

So, in answer to this section's question, even at this early stage we can be encouraged that there are some minimalist cues that will elicit useful and complex cell responses. The tough thing is to understand the language.

4.4 Tissue prosthetics and cell prosthetics – what does it matter?

It is possible to argue that the development of 'cell-scaffolds' marks a movement out of the era of tissue prosthetics and into that of cell prosthetics. As we discussed in Chapter 1, we can now produce excellent artificial hip joints made of metal and plastic. They carry out the function that was once performed by the natural tissue. However, they never work better than when they were first fitted, as they cannot repair, renew or regenerate.

It is possible to view the TE scaffold as a *prosthetic repair tissue* – in other words, a structure that supports and enhances the natural repair process. As such, its role is temporary, lasting only during repair. Whatever the finer philosophical points, the key to the prosthetic versus engineered implant difference lies in the idea that a good TE 'scaffold' disappears as a natural tissue appears, whereas the ideal prosthesis lasts as long as possible. Such a big difference demands maximal attention.

Fortunately, we have a BIG clue to keep us on track. Where the central tenet, of '**essentially temporary**' is allowed to move down the *must-have* priority list, then we return rapidly (with the help of familiar surgical imperatives) into the world of prosthetics. This is especially true in those areas where early tissue mechanical strength is the surgical demand, such as in bones, joints and large blood vessels. Repairing or (when it becomes possible) regenerating tissues are soft, weak and vulnerable; weak blood vessels burst under high blood pressure, and immature tendons will snap when they are on the end of mature muscles. Both the patient and clinician would like mechanical function to return in hours, or at least days, rather than weeks. In the case of the vascular surgeon, however, it might not be so much what they *would like* as that their patients tend not to survive the rupture of a major blood vessel.

Figure 4.5 shows the distinction between basic prosthetics and tissue engineering logics, in the form of their intended *function* versus *time*. Although these are notional, rather than real, data plots, the point is clear as their respective dynamics are distinctly opposite. The rate of change of function over time will depend on the specifics of each example and the anatomical sites they occupy.

Nevertheless, prosthetic function inexorably declines from an early stage high point. This high point is their great surgical advantage – the patients leave and are happy. Engineered implants, by

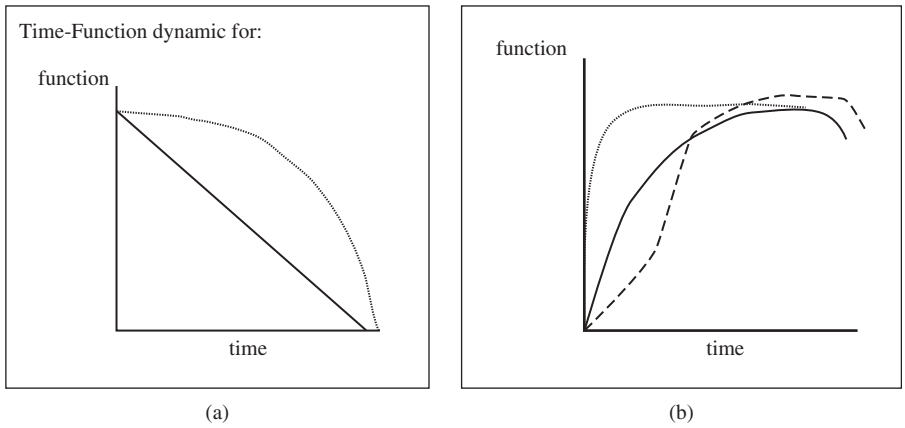


Figure 4.5 Notional (or otherwise aspirational) function-versus-time plots for (a) prosthetic implants and (b) engineered tissue implants.

contrast, are unlikely to meet local tissue demands immediately. Even perfectly matched tissue grafts would need to integrate, forming mechanical, vascular and neural links with the host tissue margins. But once that bio-integration is effective, the great advantage of tissue engineering becomes a reality – their function just goes on renewing, like other tissues.

The result of this conflict of possibilities has its effect on the respective support materials. Here there is a tendency to drift the function of *cell*-support scaffolds, for tissue engineering, such that they incorporate as much *tissue* support as possible, at time zero. This, to use the analogy of a box of chocolates, is to want all the hard centres and none of the soft, which always comes with unintended consequences.

Most commonly, we are tempted to design and fabricate very stiff, strong and long-lived scaffold materials as we mimic the function of the *finished* tissue rather than immature or repair tissue. Examples of this would be the seeding of fibroblasts onto high-tensile strength rope-like materials and calling it load-bearing tendon, or putting vascular cells on non-degradable polymer mesh tubes. The truth is, of course, that these *are* close to or actual prosthetic implants – just with added cells. Indeed, evidence now suggests that they do not ever really make the transition to the real, functional tissue that we wanted, and biomimetic engineers suspect that cells may even be getting the *opposite* signals from those we intended.

One of the best established examples of this is the use of stiff, strong cell-seeded materials in ways which stress shield the resident cells. Stress shielding is an effect where one very stiff part of the system (in this case, the support material) carries most of the overall load, shielding another less stiff component (the cells). In tissues, the result of stress shielding by stiff materials is that cells are not exposed to the physiological strains which regulate normal tissue remodelling. In stress shielded bone and fibrous tissues, cells appear to switch off tissue fabrication, repair and remodelling behaviours. This is well recognized, for example, where stress shielding of osteoblasts leads to bone loss around stiff orthopaedic implants. Certainly, many tissue cells need to be exposed to at least basal maintenance levels of regular mechanical strains (deformation) if they are to maintain or rebuild their tissue materials.

The first lesson of this example is that over-reliance on strong, stiff cell supports is perilous to good tissue engineering, even when it looks like a good idea in the short term. Viewed more positively, we can conclude that transmission of external mechanical strains to attached resident cells is an important, basic cue for connective tissue growth. Consequently, it is certainly important to

be careful when we blur the distinction between 'scaffold' materials designed to support tissues (i.e. prosthesis-like), as opposed to those which support cells. Materials providing whole-tissue support can potentially produce exactly the *opposite* effects from those we are aiming to achieve with cell supports. For this reason, we shall, from now on, remind ourselves by referring to 'cell support' materials instead of just 'scaffolds'.

4.5 Types of cell support material for tissue engineering – composition or architecture?

When we come to design our *extreme* cell support material, is it better to focus on the composition of the material or the 3D architecture we fashion it into? There was certainly a tendency in early tissue engineering to start from a point of intense faith in our favourite polymer material and work out from there. To return to our shipping analogy, this is equivalent to deciding first to build in high grade steel, aluminium, cast iron or wood, and only then to fit the cargo-type into our choice. Clearly, composition affects the eventual 3D space we can fabricate, but do we start with a promising material, or design the cell-carrying space and *then* look for the materials which are most suited?

To tackle this question, we need some basic background. First, we should try to consider the distinction between composition and 3D architecture of support materials *from the viewpoint of the intended resident cells*. Is it possible to distinguish the dominant source of the cell cues we would most like to provide (accepting that such predictions will be fuzzy)? Molecular composition, or 'the substance' of the material, will dominate many of the final properties, from its surface chemistry to its gross mechanical strength.

In fact, these two points of contact, *surface* and *bulk*, form a helpful division (Figure 4.6). Material composition is a combination of both surface and bulk properties. The first – surface properties – are typically just nanometres or, at most, microns deep. But they profoundly affect how cells bind and interact. Commonly, these act as short-term and dynamic cues. The second – bulk properties – tend to dominate the large-scale characteristics. These provide the longer lasting features, such as mechanical strength and overall gross-structure survival time.

So, for this section, we shall consider only simple 'surface-deep' support material features in relation to material composition. It is still complex, and the really fancy stuff can come later.

4.5.1 Surface or bulk – what does it mean to the cells?

Any exposed surfaces can directly affect how cells use and interact with the construct (cell binding, motility, contraction, surface degradation, etc.). Such surface chemistry presents a range of possible charged groups or hydrophobic/hydrophilic areas, or it can alter the nano-stiffness. However, it can equally act indirectly, given that almost all implanted surfaces immediately absorb a cocktail of proteins from adjacent body fluids. Some of these proteins are natural mediators of cell-binding, while others just coat the surfaces and so *reduce* cell binding by occupying space but not attaching to cells. This means that the original material surface chemistry can be selected to take up desirable cell-binding proteins.

In addition, surface chemistry can influence substrate mechanical properties at the cell level (i.e. local to individual cells – a few micrometres into the bulk material). This surface material coating, which can represent the cell world, is often dynamic in its nature. Where the material is bio-degradable and formed of proteins that are digestible by cell enzymes, it will be rapidly removed and replaced. On the other hand, the underlying bulk material, comprising the stable, major part of the material, largely determines overall mechanical strength and survival time, being unavailable to the actions of surface attached cells.

4.5.2 Bulk material breakdown and the local 'cell economy'

Let us take a closer look, then, at some examples of the bulk material compositions we have mentioned.

Figure 4.6 Surface and bulk qualities of Christmas cake (left hand panel). Bulk fruity-nutty-cake material is surfaced by a mechanically stiff, micro-porous layer (arrows). A complex sub-surface interface layer is formed by compliant, visco-elastic marzipan (arrows: right hand enlargement).

It is worth clarifying that, in the biomaterials field, *biodegradable polymers* are often 'biomimetic' only to the level of being tolerated by tissues. Many of these, in addition, break down by simple hydrolysis in aqueous fluids. In contrast, *biological materials* are formed from bio-molecules (proteins, polysaccharides, etc.), though not necessarily in native form. Finally, *natural materials* are native and tend to be derived directly from parts of animals or plants. Materials which are *not* bio-degradable are excluded here, as they seem to fail the simplest, defining test of tissue engineering. They cannot be replaced by native tissue.

A useful way of dividing cell support materials is based on biomimetic function that has become apparent in modern tissue engineering, and this identifies three generic groups in terms of their functional similarity to native tissue materials. These are, broadly:

(i) predominantly synthetic materials (synthetic polymers, ceramics, soluble glasses, etc.);
(ii) predominantly natural polymer materials (*native* proteins, peptide sequences, polysaccharides, etc.);
(iii) hybrids (composites) between (i) and (ii) having synthetic parts linked to natural domains designed to mimic some natural functions.

The logic of this classification reflects (a) the ability of some support materials to work as an integral part of the natural cell-matrix remodelling process, versus (b) those which just break down or dissolve irrespective of cell activity. This is *cell-dependent* versus *cell-independent* breakdown.

Being part of or not part of the tissue remodelling process is a bit like being a small district in central Chicago trying to live, trade and bring up families – but using Euros instead of US dollars. They cannot work, play or trade together. They are functionally disconnected – the opposite of integrated. Suddenly, this now allows us to see another great paradox. One of the most common ideals quoted by tissue engineers (in this case the biomaterials tribe) is that the 'scaffold should degrade at a rate corresponding to production of new tissue'. In other words, it needs to be quantitatively 'replaced', in a **cell-controlled** system. But how can our ideal *ever* be realized when the material degradation process is 'dollar-priced' but the cells only have Euros in the bank?

It is easy, though, to see why natural, cell-integration materials might not be the first place to start. Natural biological materials may integrate with cell physiology, but they are also difficult to fabricate reproducibly and controllably because, by definition, they are biologically complex, with possible immune and infective problems. In contrast, despite minimal participation in the 'local cell economy', many synthetic biomaterials are relatively simple, predictable and reproducible to manufacture, with a safe clinical history. This, then, is the pragmatic balance which extreme tissue engineering must resolve.

4.6 Three generic types of bulk composition for support materials

In effect, this function-based classification comes down to the philosophy that lies behind how support materials are expected to interact with their resident cells. Like any good design process, it requires an

Text Box 4.2 'Bulk' and 'surface' material properties

It is important to be clear that for many polymer materials there is a major functional distinction between the vast majority, or '*bulk*' of the material and the thin *surface* coating, which is accessible to cells (Figure 4.7). Two spatial, cell-material relationships are possible, where:

1. Cell support surfaces are essentially dense and impermeable over areas much larger than cells (e.g. hundreds of μm). To compensate for the cell-impermeable nature of such surfaces, they are formed into interconnecting pores, at the sub-millimetre (100s of μm) scale. In this case, cells normally have little access to the supporting, sub-surface bulk polymer (e.g. synthetic μ-porous plastics and ceramics). As we will see later, their high bulk : surface ratio can be reduced by increasing this μ-porosity sometimes to more than 80 per cent of total volume, with the support of even stiffer and denser pore walls.
2. Cell support surfaces are much smaller than cells (e.g. nano-fibres of hydrogels or electro-spun substrates). Such *interstitial 3D cell seeding* within the material allows cells to move around and envelope the nano-fibre surfaces. This can dramatically reduce, though never completely eliminate, the bulk-material volume from which cells are excluded.

In either case, cells only touch the outer surfaces of the 3D support material, where the 'main load-bearing elements', are (1) the single gently curving μ-pore walls or (2) the many surrounding nano-fibres.

Cell signals from the support materials seem to be a combination of surface (bio-)chemical features plus substrate stiffness, measured against the internal cell-cytoskeleton. **Surface chemical** signals (e.g. nm-deep protein layers) can interact with cell membrane receptors. However, **mechanical signals** can affect cells from much larger depths, many μm below that surface, such as soft/hard layers, gaps and splits. Curtis and co-workers[1] have compared this sensitivity to deep mechanical properties to the 'Princess and the Pea' fable, where a cell-princess feels a stiff pea through many softer layers.

For biodegradable or cell degradable support materials, of course, this relationship is, by definition, *dynamic*.

Breakdown and release of the *surface layer* potentially alters both cell-receptor and mechanically mediated cell signalling, e.g. time-dependent surface changes due to:

(a) *loss* of surface layers (e.g. uncovering deeper substance) by cell or chemical action; or
(b) by surface *re-covering* due to deposition of exogenous material, often extracellular matrix.

Hence, surface composition and properties of material supports are often different from those of the bulk material, but are disproportionately important to cell behaviour. This simple distinction has supported a large body of research into control of cells by surface modifications. Examples include synthetic polymer surface modifications, such as:

- alteration of surface charge or hydrophobicity to encourage protein binding;
- direct modification, typically coating with a tightly bound layer of active protein/peptides.

However the cell dynamic is important to remember here. Even covalently attached proteins will be broken down rapidly.

Reference

1. Curtis, A. & Wilkinson, C. (2001). Nantotechniques and approaches in biotechnology. *Trends in Biotechnology* **19**, 97–101.

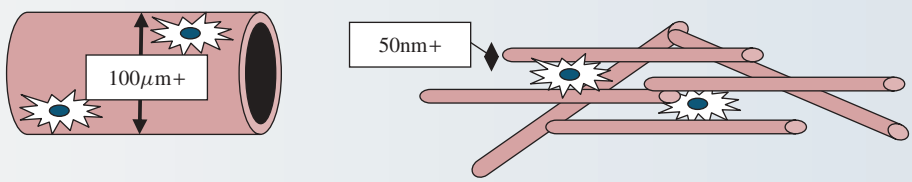


Figure 4.7 The left-hand diagram illustrates how surfaces might look/feel to cells in a type (1) relationship. They are on the walls of a deep solid material with a 'core bulk' (dark) zone with little or no role in cell signalling. In contrast, in the right-hand diagram, by changing the scale and architecture of the material to give a type (2) relationship, cells are enmeshed by the (pink) surface material with a much smaller proportion of 'excluded' bulk volume.

understanding of the practical trade-offs that each one is making. Once we understand this, we can make informed cost-benefit choices for our specific application.

Without doubt, none of these options are wrong to use but, equally, none will be appropriate for *all* applications. The days of listing the 'ideal properties of support materials for tissue engineering' in a single opening slide in a talk should now be gone. We are – belatedly, perhaps – in the era of learning the rules. Ideally, this should allow ETE to evolve into a strictly human form of ***intelligent design*(sub-division 'cell-support')**. As a result, the ability (or not) of our materials to participate in the natural cell-matrix 'economy' can become a useful defining feature of the cell-substrate combinations we investigate. These options are illustrated in Figure 4.8.

4.6.1 Synthetic materials for cell supports

The distinction between materials that cells can degrade naturally, by enzyme breakdown, and those that they cannot degrade (only dissolve) is fundamental, at least to cells. It is relatively simple to imagine the viewpoint of a cell attached to a benign, but slowly dissolving, support material. A human analogy might be your fourth day trapped in a factory making coloured-ice food novelties. You can suck on an ice-strawberry, revel in a choc ice or nibble an ice-hamburger – they are harmless, but tasteless. After four days, though, they would certainly not be giving you many of the signals of satisfying food and treats that you are expecting. Figure 4.8(a,b) summarizes the two basic modes of degradation seen in synthetic materials. These are inside-out (i.e. core first) and outside-inwards. In either case, they provide physical support for cells which could then produce new tissues.

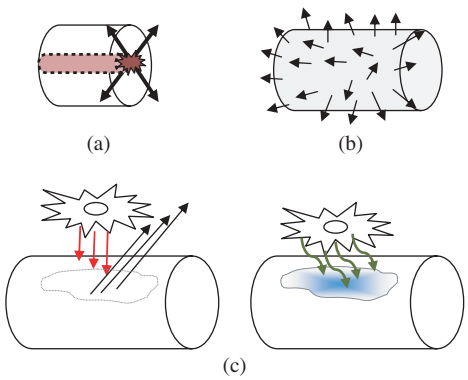


Figure 4.8 (a) Polymer core dissolution (e.g. PLGA: arrows indicate core-first loss of material). (b) Surface dissolution (e.g. phosphate glass: arrows indicate surface to core loss of material). (c) Natural polymer material (e.g. collagen protein). Attached cells secrete digestive enzymes onto the support materials (left hand diagram – red arrows), breaking down material into protein/peptide fragments which are released from the surface and lost. This leaves denuded areas around the cell footprint (dotted shape). Right hand diagram: same cells deposit new protein material (i.e. extracellular collagen-matrix – green arrows) to replace the digested material.

The first mode of degradation of synthetic bioresorbable polymers (Figure 4.8a) represents their curious habit of degrading faster at their core than at the surface, even though this is a hydrolysis process. We are more familiar with water degradation, where materials are lost from the outer surfaces, which are in close contact with the water, e.g. where river torrents wear into soft bedrock, salt crystals dissolve and steel rusts and flakes away.

A common example of core-first degradation is the copolymer poly(lactic-*co*-glycolic acid) (PLGA). This is formed from graded mixtures of poly-lactic acid and poly-glycolic acid, both of which hydrolyse in water but at different rates and patterns. Blending of the two in a range of proportions produces bulk materials with a range of compromise properties. The poly-lactic and poly-glycolic acid backbones break into their organic acid constituents (lactic and glycolic acids) on reaction with water. This reaction is dependent on water access, but the hydrolysis also accelerates at low pH. Consequently, as water seeps into the core and generates an acidic core pH, this leads to faster local breakdown far below the surface.

For high load-bearing structures such as PLGA sutures or bone-pins, the effects of this can best be seen in their strain-time profile. Strain under a sub-fracture load remains constant for many weeks or months as the core degrades but the outer shell carries the load (Figure 4.9). Then, quite suddenly, the structures will fail as the outer crust fractures,

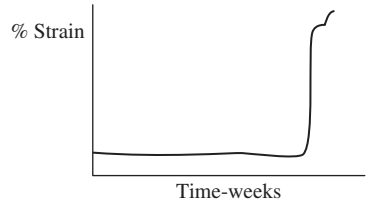


Figure 4.9 Diagrammatic plot of strain over time for PLGA (PLA or PGA) under a sub-fracture stress load. This shows the low deformation (percentage strain) of the material, constant for some months, followed by sudden and complete (catastrophic) failure.

incidentally releasing the inner degraded 'goo' of organic acids. At the multi-micron level, cells are growing on a stable, high-stiffness substrate for long periods, proliferating and laying down matrix. Then, suddenly, at some time point depending on the type and thickness of the PLGA fibre or strut, this constantly stiff cell support undergoes mechanical failure, instantly transferring major loads onto previously shielded cells and matrix. There have been concerns about the sudden release of acidic core degradation products (goo) onto cells, but in practice this, and any associated inflammation, seems to be quantitatively modest.

Figure 4.8b illustrates a more familiar pattern of aqueous dissolution of materials. In this case, the example is soluble phosphate glass. Phosphate glass is literally a glass-like material in which the silica component has been replaced by phosphate. The resulting materials react with water in complex manners to form soluble phosphate products, but in a strict outside-in direction. In other words, there is a constant loss of surface materials to the aqueous media as surface molecules of the glass dissolve and wash away. These can have rapid dissolution rates which can be reduced in a controlled manner by addition of trace contaminants to the bulk composition, such as iron or manganese. 'Bioglass' materials developed for hard tissue replacement are similar, but retain a substantial silica content and so are very slow to dissolve.

In both these cases, material is lost from the outer surfaces which are exposed to an aqueous environment. This, incidentally, means that release of these inorganic ions is proportional to the surface area-to-volume ratio (hence simple architecture). This outside-to-in pattern of dissolution has advantages, not least the gradual release of dissolution products, as opposed to the 'all at once' sudden release for PLGA and others. However, a molecular layer over the surface of such substrates is constantly being removed and released, exposing deeper levels. Where this action is rapid, it can play havoc with cell or protein adhesion.

This, then, is an example of a major affect of bulk composition on surface properties, where proteins and cells are constantly re-attaching at rates proportional to the material dissolution. Attachment of engineered cell layers, such as epithelium, to the surface of such materials is likely to be temporary (see Text Box 4.2).

Both of the outside-in and inside-out degradation patterns are, however, entirely dependent on the presence of an aqueous medium. They are biodegradable only by virtue of the fact that they lie in an aqueous environment when placed into living cell cultures. In this case, the term 'bio-degradable' does not imply any linkage or deeper dependence on the proximity of the living part of the system; they merely share the same water-based environment as the cells.

A key factor here is that dissolution is relatively simple to predict. In any definable system, the rate of loss of mass from the synthetic material will be dependent on standard physico-chemical factors, including the chemistry of the hydrolysis process, local pH and temperature and reactant equilibria (dependent on diffusion and mixing rates through, out of and away from the material), etc. However, the term '*relatively*' should have rung alarm bells, particularly for biologists. The basic physico-chemical drivers are, indeed, calculable, but not necessarily with any accuracy for dynamic and spatially complex bio-systems. So, while it is true that dissolution and failure rates for synthetic supports can be modelled and predicted for cell-free, and some simple cell, culture systems, predicting the fate of this group of materials after implantation *in vivo* is quite another fish market.

In contrast, biological materials (Text Box 4.3 and Figure 4.8) work in a completely different manner. Figure 4.8c illustrates the starting point for this contrast. Specifically, native biological materials can be degraded by embedded cells and, in some cases, cells will also add new matrix to fill in the holes made by this degradation. Because it is now becoming clear that this difference is so important, our summary of these material types will be unusually weighted – short on synthetic, long on native materials. This is the reverse of many tissue engineering uses past and present, but it may better explain where it will go in the future.

4.6.2 Natural, native polymer materials for cell supports

We have made a great deal of the ability of cells to 'use' and 'remodel' the bulk material of our synthetic or native support materials. In our own lives, the slow, inevitable degradation of the fabric of our house might leave us feeling anything from mildly concerned to panic-stricken. Where we are on this spectrum is determined by our repair skills and access to suitable tools and materials. For those of us who are handy with a hammer and a screwdriver and live close to a DIY store, it is an opportunity to improve and customize our living space. Steering clear of the dangerous practice of humanizing cells, we can see from this analogy that **the (chemical) nature of the bulk materials *can* have a greater impact on later 3D structure than expected**.

This is important enough to merit a second – short time-base – analogy. We have all probably eaten ice cream cones. Some cones have the cold stuff (plus chocolate and sweet bits) tightly wrapped in a colourful cardboard-and-foil cone. These are jolly, entertaining, insulating, easy to grip – but inedible. The other type is squashed down into a biscuit cone, which you can eat. Most likely, your relationship with the first, card-cone-support system is simple, consisting of: (i) a brief pleasure-support role, (ii) tearaway moment (optional), (iii) splat flat, then bin. The biscuit cone elicits a completely different set of interactive behaviours. You eat the biscuit as the ice cream is consumed, in parallel, partly nibbling *as you go*, snapping off portions to scoop up ice cream, reshaping and remodelling *as you go*, mixing and repositioning the ice cream flavours, choc-chips and sugar sprinkles *as you go*.

Critically, different types of people (just like cells) have their own behaviour patterns for this coupled spatial remodelling process. But, at its core, the behaviour is transformed by *your relationship* with the material; in particular, your ability to consume the biscuit (or cell-support material) dominates when, where and what you do. The point here is that we should expect the composition of native materials to have a greater impact on final tissue structure than that of synthetic materials.

Where natural material composition is concerned, the need for physical strength and bulk volumes largely excludes all but two natural polymer groups – proteins and polysaccharides. Generic examples of fibre-forming, aggregating protein materials are relatively small in number. Most work has focused on collagens, fibrin, silks and fibronectin – but that is about all the choice that is available. It is worth noting the obvious, that all silks differ from the other three types in *not* being vertebrate proteins (Text Box 4.3).

Many **polysaccharides** are available, but even fewer of these are mammalian. Polysaccharide aggregates can be given significant mechanical strength, either naturally or by chemical processing, and so are common as cell substrates. Examples include starch, agarose, chitin (chitosan) and hyaluronan (or hyaluronic acid). Starch is a glucose polymer whose properties, from dough to pasta, are completely familiar to us, courtesy of bakeries, patisseries and pizza suppliers. Agarose is a galactose-based refined seaweed product (algae, hence 'alginate') which is commonly used in biology to form water-rich (hydro) gels. These handily undergo sol-gel transitions at temperatures which can allow cell incorporation (on cooling). Chitosan, a chemically de-acetylated, poly-D-glucosamine, a derivative of chitin, is made from crustacean shells (i.e. a by-product of the shrimp industry, chiefly *Pandalus borealis*). Again, it can be made into tough polymer materials with controllable 3D structure.

Text Box 4.3 Biological: but is it native, non-native or biomimetic?

For non-biologists, the apparently mild tautology of 'native biological materials' may have caused gentle irritation. Actually, though, it is not self-reinforcingly obvious, and the reason why is worth explaining. It is arguable that almost all living cell products are 'biological', as they were formed by living systems. Their origin dominates. However, it is equally clear that some have been processed and modified so far from their origins that they take on quite different properties, and so deserve a different name.

For example, wood may be dried and seasoned, but remains much the same, such that it can undergo rotting by microorganisms, much as it would on the forest floor. However, when pressure-injected with creosote fixative or shredded into an embedding resin (e.g. chipboard), it has radically altered stabilities. Similarly, we are keen to distinguish between cell-free sheets of native collagen material (e.g. dermis, fascia, **s**mall **i**ntestinal **s**ub-mucosa – SIS) and leather, which is strongly cross-linked by tanning. Such processing is designed to divorce the biological material from natural bio-degradation processes such as bacteria or fungal action or digestive enzymes. This makes them much more stable for everyday use.

We would say, then, that such processed biological structures were no longer 'native'. This is a particularly important distinction in our subject as, by definition, native materials *will* be available for cell-mediated degradation and remodelling via cell enzymes, but non-native biologicals will not. Chemical cross-linking (e.g. with glutaraldehyde or carbodiimide) of collagen materials, like leather production, renders them both resistant to remodelling and non-native. They are, then, fairly questionable candidates as tissue engineering supports.

An interesting parallel distinction lies in the term 'biomimetic', since, like 'biological' this is a term which is far more useful once qualified. Designing a material as a copy of *any* bio-system or structure *can* be biomimetic. But in biomedicine, we are normally interested in mimicking at least mammalian – and preferably human – systems. We are less bothered about bacterial slimes, crustacean limbs or plant cell walls. Hence it is helpful to specify what, in general, is being copied. This rigour makes it easier to ask what part of mammalian biology is mimicked, for example, by alginate seaweed gels or chitin? (See also Chapter 2.)

Hyaluronan, formally known as hyaluronic acid, occurs widely (though rarely at high levels) in many mammalian tissues, and so is bio-medically mimetic. It is a strongly anionic polysaccharide, composed of repeating disaccharide units of glucuronic acid and N-acetyl glucosamine, making it a member of the glycosaminoglycans family. In its native form, it is strongly hydrophilic, forming very long but unbranched, randomly folded chains. Its composition and charge means that it binds many times its own mass of water to form gels or highly viscous fluids. It has also been processed at an industrial scale into more stable and physically strong materials by progressive levels of cross-linking. These are formed into sheets and fibres, and some are in current clinical use, notably as perforated sheets for the support of dermal repair.

In fact, hyaluronan materials are particularly useful for illustrating the rather typical compromise spectrum common in biological materials – that of increasing modification versus loss of native biomimetic properties. The problem here is that, like many polysaccharides, hyaluronan chain structures do not really self-associate in any organized manner to form a solid aggregated material (in contrast to proteins, below). As a result, the 'solid' material structures frequently have indifferent or really poor material properties.

Unmodified hyaluronan, for example, falls somewhere between a weak gel and a viscous fluid, depending on its water content. This, of course is the native format, where it is most biomimetic. To 'improve' its physical properties, hyaluronan is commonly cross-linked by chemical treatments to produce useful solid materials. Unfortunately, as the extent of cross-linking increases, the ability of cells to use, digest and remodel it is gradually lost. This is similar to how the tanning process turns biological skin collagen into leather, which is impervious to cell enzymes as well as to bacterial decay. As a result,

hyaluronan materials, with low level cross-linking, remain biomimetic but degrade faster and are less strong than highly cross-linked forms.

A small number of **natural proteins** can usefully be fabricated into practical biological support materials: the collagens, fibrinogen/fibrin, fibronectin and the silks. On the whole, these have mechanical and support functions in nature, either at the cell-support or the gross-tissue levels. An interesting common feature of all these is that they are able to self-assemble from relatively low molecular or monomeric forms. All of them form fibrillar materials by side-to-side aggregation and the axial accretion of many thousands or millions of monomers. Since the component monomers are in the nanometre scale and the gross materials can be metres in length, overall aggregation is not limited, though the component fibres can be, lying in the nm–μm range of diameters.

Again, a common feature is that fibre elongation can be driven by application of fluid shear forces (producing shear-aggregation or pseudo-liquid crystal behaviour). In other words, the directional shear of moving fluids tends to extend and elongate the protein material as it aggregates, potentially elongating and aligning fibres, like spinning candy-floss though air. The value of shear-driven aggregation in water-rich systems is not surprising as it simultaneously expels water and aligns the long, thin molecules, physically packing them close together. In many cases, fibre formation can be defined and driven by this processes of **aligning dehydration**.

Flow alignment also supplies us with a useful example of how natural material fabrication might occur Figure 4.10. Development of **silks** springs partly from their remarkable material properties and long track record as suture materials. Much of the modern focus, especially on their use for tissue engineering, has grown from work of Vinney (2000) and Volrath & Knight (2001). Their dream is to understand how spider or silk-worm spinnerets achieve shear-aggregate fibres from the fibroin protein monomer.

Biomaterials and tissue engineering companies have formed around technologies for using silk-like materials towards applications as diverse as nerve

Figure 4.10 Silk fibres being extruded from spider spinnerets. Reproduced with permission © 2004, Dennis Kunkel Microscopy, Inc.

regeneration conduits, cartilage and bone replacements. These are elegant developments, using subtle biomaterial modifications which initially seem strongly biomimetic. However, the counterbalance to their safe history is that silks are proteins from a *very* different animal phylum – the Arthropoda. So should we ask how much the silks mimic **mammalian cell systems** and, before lavishing cost and effort on engineering silk materials, determine if there are more appropriate mammalian proteins we can use?

Two mammalian blood-plasma protein candidates are fibrinogen/fibrin and fibronectin. Each has been as a 3D material or components of materials in significant numbers of applications. Both are human in origin (even potentially autologous, from the patients' own blood), and available in industrial quantities through the plasma fractionation industry. This means that they are non-immunogenic and, even though they can carry human pathogens, these are already assessed and cleared by the plasma fractionators, so are as safe as other human plasma products.

Fibronectin can be (shear-) aggregated from bulk solutions of the native protein to give dense fibrous materials. This process may be similar to that used

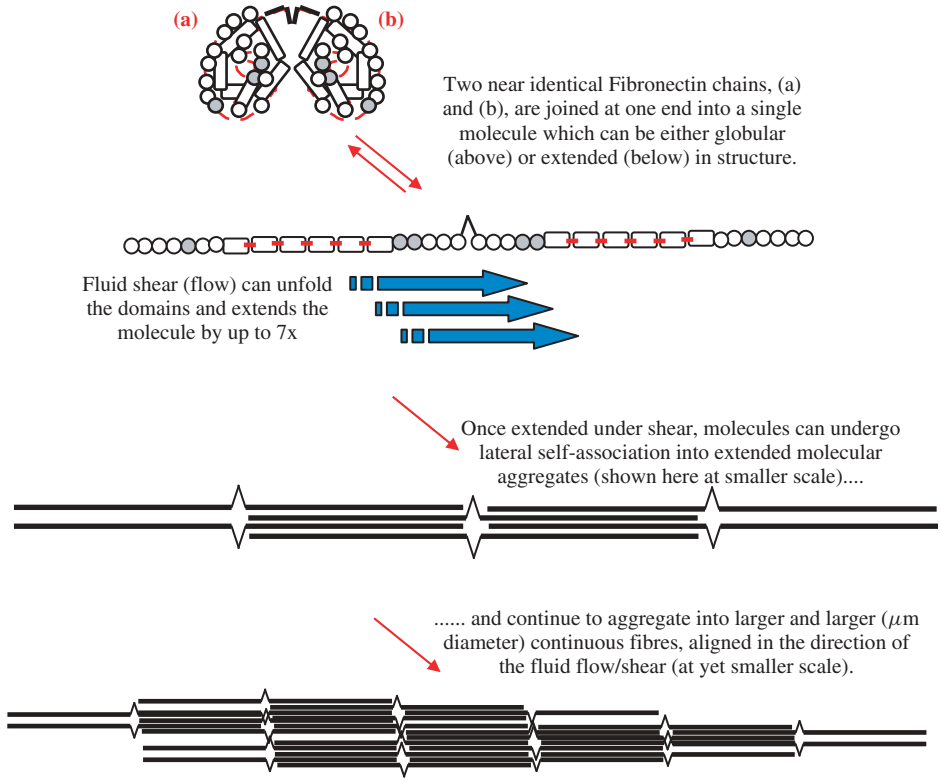


Figure 4.11 Diagram illustrating how some proteins (e.g. fibronectin) may extend and aggregate under directional fluid shear – shear aggregation – to produce fibres and fibre alignment. This mechanism predicts that aggregation will be enhanced where molecules and fibres become anchored to fixed points and where fluid viscosity increases.

by cells in nature to aggregate fibronectin fibres during tissue repair, growth and cell migration. Fibronectin shear-aggregation involves stretching out of the native molecule by application of fluid shear (Figure 4.11). This changes it from a globular to a rod-like molecular structure, and so promotes lateral packing into fibres. Once aggregated, the bulk *and* surface properties are highly biomimetic and ideal for cell adhesion and guidance. Indeed, fibronectin is exactly the substrate deployed naturally in the early stages of tissue repair for exactly these functions. However, as always, excellence in cell guidance comes with a cost and fibronectin materials have limited gross physical strength. Consequently, applications so far have been restricted to promotion of guided nerve and spinal repair through guidance conduits. Many other applications could benefit from this bulk material as a integral component, just as fibronectin monomer is widely used to surface-coat synthetic materials.

Fibrinogen/fibrin is already available as a self-assembling clinical material, sometimes known as 'fibrin glue' or 'fibrin sealant'. Fibrinogen is the soluble monomer precursor fibrin matrix in everyday blood clots. When our blood (or the cell-free protein liquid part, called plasma – Text Box 4.4) clots, the *coagulation cascade* is activated, (long enzyme pathway – not relevant here) This leads to formation of active thrombin, a protease which rapidly clips off specific tail-sections of the soluble fibrinogen protein, producing fibrin. Fibrin immediately self-aggregates into a fibrillar gel, which then progressively cross-links and shrinks to form a dense gel-material.

Surgical fibrin glues have been commercially available for many years, with separate fibrinogen

Text Box 4.4 (Vampire) trivia for non-biologists: blood, plasma and serum

Oddly enough, our most dramatic of body fluids is handily colour-coded. Whole blood (cells plus liquid) is, of course, red. However, this colour comes from the red blood corpuscles and, if we remove the cells, the remaining thick protein solution is *yellow* (straw-yellow, rather than canary-yellow, for the more discerning). However, it matters *how* you get rid of the cells. When we centrifuge cells away in an anti-coagulant (i.e. no clotting), we get *plasma*, which includes its original fibrinogen and coagulation factors. If we are cheap and just let this clot (allowing the fibrin and cells to form a solid clot), we get the yellow liquid comprising the blood proteins *except* for the fibrin, etc., used to promote cell growth in culture. Just to be confusing, fibrin clots alone are pure *white*!

and thrombin solutions 'ready to go' in two conjoined syringes. The two liquids are mixed and expelled when the plungers are pressed, so that the fibrin gel sets at the point of application. Tissue engineers have found this trick useful, as have surgeons repairing blood vessels, urethra and intestines, where it is also important that the joints do not to leak.

Such fibrin glues clearly also represent a handy means to produce 'instant' cell supports which can be assembled as and where needed. Importantly, the fibrin gel forms around the desired cell population and may also be injected directly into tissue spaces. Fibrin glues are sometimes loaded with drugs or protein growth factors to assist cell function, with the obvious advantages of a rapid, convenient and off-the-shelf system. In addition, traces of coagulation factor XIII (also known as plasma transglutaminase) cross-link the fibrin fibres, increasing the gel stability, while fibronectin (also a contaminant) is incorporated and assists cell attachment and migration. Less obviously, polymerization by a physiological enzyme means that cell seeding is at the time of fibrin-fibre formation. Cells are trapped, right from the start, within a meshwork of fibrin fibres, just as they would be in the body.

This is major. Put another way, the time needed for cell-seeding and infiltration is *zero*: there is *no* cell-seeding stage.

Unfortunately, free lunches are rare events, even in this branch of tissue engineering, and fibrin-based support materials have their own limitations. Being gel materials – albeit dense gels – they are inherently poor in load bearing. In particular, evolutionary pressures do not seem to have driven fibrin clots to develop substantial tensile or shear properties, or to hold sutures well. In addition, most repair sites have the cell and enzyme machinery to digest away fibrin, leaving it with survival times of only days or even hours.

Typically, then, fibrin-based cell supports are either excellent in directing (certain) cell functions and timed events, or alternatively, they elicit wholly inappropriate biological responses. Examples include the distinctive patterns of cell adhesion and migration, supported by fibrin. Specifically, keratinocytes attach poorly to fibrin and tend to grow *beneath* a fibrin layer. On the other hand, thrombin and fibrin degradation products have significant downstream biological effects on fibroblasts. These are powerful and biologically pre-programmed effects which may or may not be welcome as we engineer tissues.

The final family member, **collagen**, is one of the most widely used tissue engineering materials of all. It may also be the most misunderstood and confusing. It, too, self-associates into fibrils, sometimes with help from shear or enzyme action. However, its main route to fibril aggregation is more 'crystal packing' than 'enzyme drive' and it has more in common with silk than with fibrin. Essentially, at physiological pH, temperature and ionic strength, the distribution pattern of surface bonding along collagen monomer molecules matches *so accurately* that molecules stack together side-to-side. However this only happens when each touching partner monomer lies one quarter staggered to its

neighbour. As a result, collagen self-aggregates naturally to give *very* long, strong and tightly packed fibrils. The basis of its strength is the highly regimented molecular packing implied by the strict quarter-stagger. Each new monomer can bind into a growing fibril in just one position, relative to its nearest neighbours. This positional dictatorship comes from the exposed amino acid sequence of each molecule and their limited ability to bend and wiggle (hence, 'no wiggle room'). Such a semi-crystalline molecular packing is the characteristic of collagens and silks, producing strong fibrils, distinct from those of fibrin and fibronectin.

Collagen materials and cell-supports have been fabricated in many physical forms, using a range of processes, and this generates a good deal of confusion. It would be more accurate to consider the term 'collagen materials' as the name of an entire club rather than that of one of its members. As a rule of thumb, collagen sponges are very different to collagen gels, and in both cases there are native, cross-linked, soluble, insoluble and aggregated forms. For most of these examples, though, the differences are mainly of structure rather than composition – in other words, beyond the subject of this chapter (but see Chapter 5). To stick to the topic of composition, we can divide collagen materials into four generic starting materials (predominantly animal-derived type I collagens). These are:

(a) highly cross-linked, insoluble collagen (e.g. reconstituted from shredded suspensions);
(b) tropocollagen (native monomer);
(c) atelocollagen (enzyme-extracted, monomeric with small, key end-sequences cut off);
(d) gelatine or heat-denatured collagen.

This series excludes two much quoted natural 'materials' *containing* collagen:

(i) decellularized whole animal tissue preparations (e.g. small intestinal sub-mucosa-SIS); and
(ii) matrigel, a basement membrane analogue, rich in type IV collagen, from cultured tumour cells.

Both are left out here as they are not fabricated from extracted collagen (bottom-up) but by simplification and processing of whole animal or cell products (top-down cultivation). We will return to this later, but essentially it is the difference between starting complex and working to make something simpler, versus building up complexity from simple starting components. The bottom-up-top-down fabrication distinction (Figure 4.12) is essential here, partly as it defines a central spit-line in biomaterials fabrication. Also, including top-down natural materials could bring all connective tissues and cell cultures, however heterogeneous and poorly defined, to the same discussion.

To elaborate further on the four types of collagen-derived materials:

(a) *Insoluble collagen materials* have a long history as haemostatic sponges and tissue supports. They can be as crude as homogenized tissues, reconstituted and freeze dried, but even these processes remove many original tissue components, such as cell debris and non-collagen proteins. In these materials, the structural elements are made up of shredded fibre bundles, mostly in the sub-millimetre size range.
(b) *Tropocollagen* is the intact, mostly monomeric, acid-soluble form. It is a promising starting point for bottom-up fabrication and readily aggregates into nanometre-scale fibrils and gels. Unfortunately, the extracted yield of this collagen from most mature tissues is low, due to cross-linking (so it is better from juvenile tissues).
(c) *Atelocollagen* is also a soluble, monomer-rich collagen like tropocollagen, but it solves the yield problem by the use of a protein-digesting enzyme (a protease, e.g. pepsin) to break down much of the tissue except for the collagen triple-helix. This cuts the cross-links by removing the short non-helical end extension or 'telopeptides' of the collagen, where most cross-links are located (hence 'A-telo-collagen'). Unfortunately, telopeptides are also important for normal fibrilogenesis, so atelocollagen is rather poor at forming gels – a bit of a death-blow for

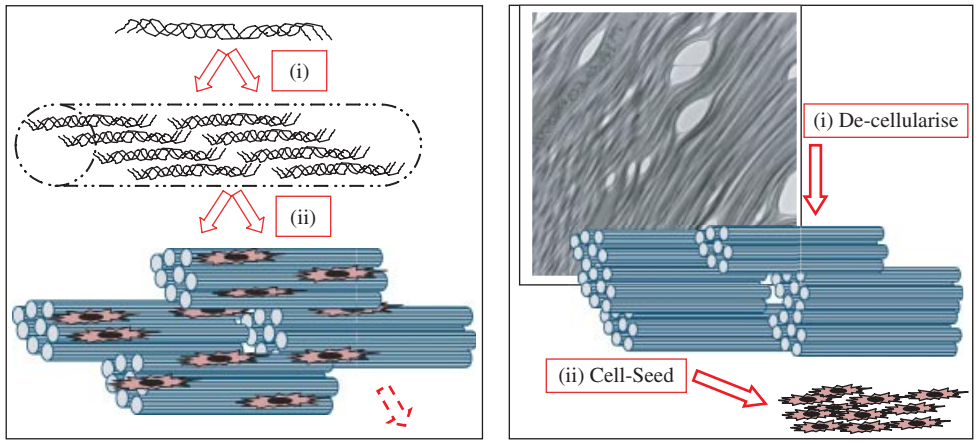


Figure 4.12 Scheme illustrating the two routes which have evolved for preparation of native collagen-based materials and cellular constructs for engineering of tissues. (a) shows the so called 'bottom-up' approach of assembling the smallest available building units which it is feasible to use. In this case, 'smallest' is collagen monomers or fibrils, with cells into the required 3D architecture. (i) Purified (normally type I) collagen monomer, acid-soluble collagen, comprises a three-chain elongate helix, 300 nm long by 1.5 nm diameter. At neutral pH and 37 °C, these spontaneously aggregate into quarter-staggered semi-crystalline structures which produce long cylindrical fibrils (≈30–100 nm diameter), trapping any living cells (or non-living particles) within their mesh. (ii) Physical expulsion of fluid from this gel produces a collagen-cell mesh construct dense enough to fabricate a simple living tissue. The 3D complexity of such tissues can be increased (as discussed in Chapter 6), again by incremental addition of components to each layer and by adding many more layers. (b) Shows the reverse strategy, 'top-down', in which a native tissue is first harvested from a suitable animal source; these can include a number of internal fascias (e.g. intestinal and bladder) or tendon and dermis. The dense collagen network (i) is then stripped of its cell content, using a range of disruptive and extraction approaches, to leave a decellularized but otherwise intact collagen material. Any immunological challenges or infective agents are also, hopefully, removed with the cells. (ii) By definition, cell seeding can then only be done after this stage. This means they are met by a dense-packed fibrillar material, where only the surfaces are available (i.e. even though cell debris can get out, whole cells are too large to get in). In effect, animals cells were embedded physiologically in the mesh (they made it around themselves), but these ≈15 μm nominal diameter cells were removed by fragmentation down to an easily extractable molecular scale. Replacement ≈15 μm human cells cannot possibly get back into the nano-fibre mesh simply by seeding. Some can, and they will, over extended periods of culture or *in vivo*, but only because they enzymatically disrupt and remodel that very special native collagen architecture which was the great advantage of this approach. This strategy has a long and successful history in *prosthetic biomaterials*. For example, replacements for defective human heart valves were developed using pig valves cross-linked, sterilized and cleared of immunogenic epitopes by glutaraldehyde treatment. While these remain effective prosthetic implants (life ≈15 years plus), their permanency is clearly not appropriate for tissue engineering.

bottom-up materials fabrication based on gel formation.

(d) Finally, *gelatine* is readily available, cheap and forms gels. However, it is made by boiling collagen until it breaks down and loses its native triple-helix structure. All gone are the triple helix, the tensile properties and quarter-stagger molecular packing of native collagen gels. In the case of gelatine gels, most of the collagen biomimetic properties are also consigned to the junk heap. Perhaps the greatest loss is that of tensile strength and resistance to enzyme degradation. Even the material aggregation process is different, as they gel in a manner completely opposite to collagen – on *cooling* rather than *warming*. In effect, gelatine gels are poorly biomimetic, weak and bio-unstable.

To summarize the above, the insoluble collagen materials family (a) are made by aggregating ready-polymerized clumps (μm scale) of collagen fibrils as their bottom-up building blocks. Soluble

(monomeric) collagens of families (b) to (d) start smaller and aim to aggregate single molecules into (nm diameter) fibrils and so gels. Of these, acid soluble tropocollagen (b) is the most biomimetic. Whilst atelocollagen and gelatine (c) and (d) get around some inherent drawbacks of tropocollagen, they trade this off against biomimesis.

Dermal repair offers us a great exemplar sequence for these types of natural material applications. Surgeons have used living skin grafts to fill up holes in their patients for many centuries but, while this has improved steadily, other top-down technologies have also developed to make:

- non-living alternatives from preserved cadaver dermis, and further from:
- animal dermis (de-cellularized and cross-linked to reduce immune reaction and infection).

An example of this is found in Permacol™, a sheet of porcine dermis, treated to remove cells, chemically cross-linked and then washed and dried. Cadaver and **top-down** processed tissues such as these have helped address the problems of the shortages of, and inability to store, graft tissue, and have also helped to avoid inflicting further wounds on already sick people at the donor site. In parallel to this, though, we have seen the emergence of **bottom-up** approaches based on soluble and insoluble collagen starting compositions (i.e. low and not-so-low bottom-up).

Integra™ (or collagen-GAG sponges) forms a good example of not-so-low bottom-up dermis fabrication. Shredded insoluble collagen is bonded with traces of glycosaminoglycans (GAG: sulphated polysaccharide) by extreme dehydration. This gives a stable, native porous collagen sponge which can be implanted to recruit local cells and support dermis replacement following burns or trauma injury, just like a skin graft. However, while Integra™ mimics dermal collagen and stays around to fill in the patients' gaps for long periods, it does not contain or deliver cells (the drying/bonding step guarantees this, as it is cell-lethal).

Enter soluble collagen (tropocollagen) gels, containing human cells which promote local healing. Apligraf® is a native, acid-soluble collagen gel, already interstitially seeded with human dermal fibroblasts to improve and speed up dermal repair. As part of its production, the initial cell-collagen gel is cultured for some days to allow its fibroblasts to contract and strengthen what was initially an extremely weak gel, producing one which can at least be handled. So we have here an example of both insoluble collagen *and* soluble tropocollagen gel implants. Interestingly, where each is biomimetic, they still straddle the same compromises. Integra™ is a strong and durable support for endogenous repair, but without its own cells, whilst Apligraf® is mechanically weak and remains only a few days, yet delivers repair-enhancing cells and growth factors in that time.

Bottom-up collagen processing may have received a technology boost in recent years, to break this compromise loop. The big problem with processes which use resident cells to increase collagen fibril density of gels is that they depend on cell forces to expel water. This is slow and costly but, most of all, the forces are too small to produce the material density we need. Native collagen gels start at <0.5 per cent collagen (in fact >99.5 per cent water). After cell contraction, we can only get this as high as 1–2 per cent collagen (still 98–99 per cent water), which remains extremely weak compared with dermis (17–20 per cent collagen).

This huge strength gap has now been bridged by a technology called collagen plastic compression. In this process, controlled amounts of the excess water are rapidly forced out of the hyper-hydrated cell gels under external load. The whole process takes minutes, rather than days, and it leaves the cells unharmed (indeed, with nothing to do). It produces a tissue-like construct of any required density up to 30 per cent collagen, and makes it feasible to fabricate strong collagen-based 'tissues' *around* the cells (Figure 4.12a), without asking them to do anything.

The previous biomimetic compromise is gone and, incidentally, the process is an order of magnitude faster. Viewed in this way, direct fabrication technologies could eventually impact on tissue

engineering in ways similar to the way transistors once changed electronic devices.

4.6.3 Hybrids: composite cell support materials having synthetic and natural components

Over the last decade in tissue engineering, there has been a gradual dilution of the early view that synthetic cell-supports represent the route of choice for reasons of reproducibility or regulatory approval. This has been accompanied by an increasing willingness to consider the benefits of biological or natural materials, and so by implication the need to work to solve their disadvantages. Central to this is the growing understanding that cells need to recognize and fully utilize the support substrate if we reasonably hope to achieve good biomimesis. Part of this movement has found its output in the development of novel families of hybrid support materials with both synthetic and biological – or at least biomimetic – components.

The initial logic of hybrid cell support materials springs from a large body of experimental effort to improve the cell attachment and utilization properties of synthetic polymer supports, most commonly PLGA. This started with coating the polymer material surfaces with recognition peptides for the cell attachment receptors known as integrins, most commonly RGD(S) peptides (Text Box 4.5).

At some point, a research movement of 'Scientists for Cell Attractive Substrates (SCAS)' was formed when a group of biomaterial scientists got together with a breakaway group of the developmental biologist tribe of tissue engineering (possibly in one of the better bars in Zurich). They got to discussing the mimicking of processes from early embryonic developmental as an approach to engineering tissues.

The theory they produced runs something like this: 'We now know a whole mess about growth factors, signalling cascades and cell phases/movements which are involved in 3D embryo growth, so let's recapitulate *bits of it* for tissue fabrication.' We can now only guess at the conversation that led these two groups to hatch their vision. The rather hazy legend has it that it started like this:

Biomaterial scientists: *It's been a rubbish month in the lab. We coated all our scaffolds with every kind of RGD peptide. Some were hanging off on long spacer-arms, while some were tight bound to the polymer. All we got was 5 per cent cell seeding, and* ***they*** *dropped off the surface and died in a week. To make it worse, the 95 per cent that fell off onto the support dish self-organized into a neural network and have started planning chess moves.*

Developmentalists: *We know what you mean. We got just the same neural net to form in monolayer, but it flatly refused to suggest any more than three moves ahead until we put it into a 3D apartment block with options to remodel the upper floors.*

So, the two groups formed a **joint research committee** and set to work making polymer scaffolds which cells could attach to and move through by degrading the matrix substance. At the same time, the moving/remodelling cells would release factors useful for controlling the arrival of other cells (perhaps from the next building). In effect, the

Text Box 4.5 Engineering with peptides to hold cells down

In the main, cell-matrix binding acts through a family of cell membrane receptors (the integrin family). While cell membrane integrin expression can be quite distinct between different cell types, many recognize and bind to very similar short amino acid sequences on the most common matrix proteins. The functional part of these peptides frequently has the sequence arginine-glycine-aspartic acid (+/− serine), hence they are known as RGD sequences. Another integrin recognition sequence, YIGSR, is commonly associated with basement membrane attachment sites. It has long been a dream of tissue engineers to use these peptides, attached to biomaterial surfaces, to improve or control how cells attach.

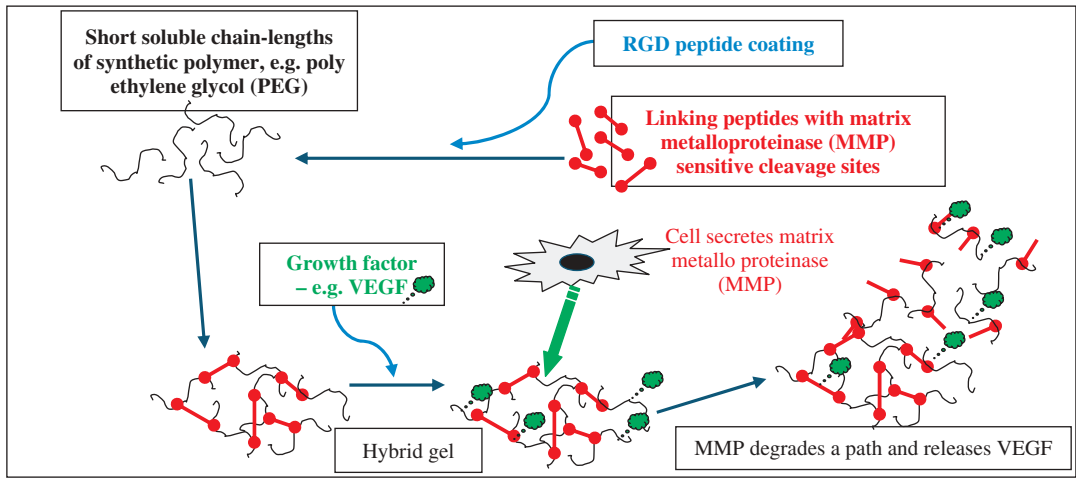


Figure 4.13 One example of how a hybrid gel of increasing complexity might be built up. Soluble PEG chains, potentially with RGD peptide coating, are linked together by peptide sequences such that the complex forms a hydrogel matrix. The peptide link sequences contain protease sensitive sequences which allow cleavage by cell-derived MMPs. With incorporation of the growth factor VEGF to stimulate angiogenesis, this gel becomes a designer substrate for local cells to move through, degrade and attract blood vessel in-growth (Seliktar *et al.* (2004) and Lutolf & Hubbell (2005)).

design of this is a simplified version of how we understand natural cell-matrix systems might work (Figure 4.13).

Unlikely (but memorable) as this fiction might be, it illustrates the core philosophy of this emerging group of *hybrid* cell support materials. The concept of how to go about the hybridization is based on linking ever greater varieties of modular parts (biomolecules, such as peptide recognition and cleavage sequences, growth factors) onto a structural polymer backbone. Cell-specific matrix cleavage is achieved by linking short support-polymer sequences together through synthetic peptides with a cell-enzyme cleavage site (e.g. in Figure 4.13, the ubiquitous matrix metalloproteinase, MMP-2). This linking is designed to aggregate the complex into a 3D cell support (normally soft), which may also carry RDG attachment sequences. Cells moving over or into such matrices naturally secrete proteases and so also degrade the support as they move.

A second thread of developmental biomimetics has evolved in this system involving cell-signalling growth factors. These speed up (or slow down) other cell processes which are needed to assist the new tissue formation. A prime example of this is to encourage angiogenesis or blood capillary (micro-vascular) in-growth for bio-integration and nutrient supply. Favourite amongst the factors used are vascular endothelial cell growth factor (VEGF) and fibroblast growth factor (FGF). Alternatively, platelet-derived growth factor (PDGF) and/or transforming growth factor-β (TGF-β) are candidates to promote connective tissue formation, for example in dermal tissue engineering. Such systems, though, can need a great deal more understanding of bio-control than we possess, in terms of the combinations, quantities and sequences of factors needed to achieve any given response.

Since many growth factors need to be free to leave the material and enter the target cells, physiological growth factor binding molecules have been coupled directly to the support polymers. These binding molecules then hold growth factors onto the support material until it is removed by an adjacent cell. Other cells can take up growth factor signals from the surface of the material, as they do in nature. In one example of this (Figure 4.13), heparin, a natural binding molecule for a family of growth

factors, is chemically immobilized to the polymer matrix. Simple mixing of the material with heparin-binding growth factors, such as VEGF or FGF, loads the support matrix with control factors, which are now accessible for uptake by cells. Such direct cell-uptake would not be possible if the factors were directly bound to the matrix.

Such biomimetic surface and bulk modifications now present the possibility for full blown hybrid biomaterials where synthetic polymer supports are given complex cell-responsive functions. This tackles the difference between synthetic and natural-biological cell-supports as described in the previous sections (4.6.1 and 4.6.2). By making the synthetic polymer and peptide sequences in a manner which forms gels on mixing, it is possible to trap cells within the matrix as it forms (interstitial seeding). This also creates a matrix which it is possible for cells to degrade directly with their enzymes. These represent (semi-)synthetic analogues of native matrices such as collagen and fibrin as described above. The example illustrated in Figure 4.13, from the Hubbell research labs, is based on soluble polyethylene glycol as the synthetic backbone, but others are possible. Many of the component ideas are based on earlier work using fibrin materials or conventional surface modifications of PLGA materials.

In theory this leads us to a 'Lego' type of system for building up and tailoring biologically mimetic support materials to match any given local cell/tissue application. Sadly, no one has really reported on chess-savvy cell networks, so we cannot test the original myth against a rated chess master.

Excitement generated by hybrid materials is real and justified, though it is important to ask where they lead in practice. They clearly represent new tools to understand in detail the key cell events and molecular sequences which are so important to tissue formation, especially in 3D. However, as tissue engineers, it is critical for us to know if their utility is in *understanding how* tissues are formed or as practical cell-support materials *ready to apply* to implant fabrication.

One such analysis is to compare the advantage/disadvantage profile of the hybrid itself with the two hybridized elements. **Synthetics** are reproducible, cheap, easy and safe to produce, but are poor biologically. **Natural** materials are in many ways as good as we can get biologically, but they can be difficult to produce and their very naturalness raises questions of safety and predictability. The hybrid systems can be judged on the balance of how much they bring of the *best* or the *worst* of their components.

It seems likely that hybrid matrices are presently more research tools than imminent, practical implants. This is partly because of production cost complexity and partly as our understanding of the workings of cell-matrix systems is still rather too simple for us to assemble the best 'Lego' polymer parts. In addition, sharper readers may have noticed that the discussion of hybrid materials so far has focused exclusively on composition. Spatially when, where and how things happen, and the control of material-mechanical properties, μ-shape, asymmetry and direction, are hardly yet on the agenda. Thus, our analysis might suggest that hybrid materials have lots of practical potential for tissue engineering but are longer-term prospects for clinical applications. There is a strong probability, however, that they will inform how we design and fabricate the compositions of ever more biomimetic implants.

4.7 Conclusions

Tissue engineering relies heavily on the idea that substantial control can be exerted on cell processes through the surface and bulk (composition) properties of its support materials. In terms of options, there are three clear general concepts on composition: synthetic, natural and a hybrid of the two. Despite the enormous spectrum of combinations, this division is helpful as it allows a rational analysis of the *functional* cost benefits for cells in any given application. The task of the tissue engineer remains to develop supports which have *biomimetic* and *dynamic* cell control properties for the cells and site (or bioreactor) where they are to be used. Meanwhile, next-generation cell support materials

will reduce the major compromises we presently have to make, while hopefully avoiding the prosthetic logic. In particular, this means identifying the *key* biological processes that we need to promote or to shut down.

In recent years, the pendulum has probably swung away from the dominance of traditional, unmodified synthetic polymer support materials. Natural (usually protein) materials have attracted greater interest, but a great deal of basic work is needed to understand how to fabricate these to the level needed. In this, we are learning more from how natural protein fibres are produced in natural systems, particularly the relative perfection of their packing, and how they influence cells at so many levels. Hybrid matrices, may provide major clues as to what is needed for better natural and synthetic support materials by showing what controls are *really* important (and when) for any given tissue engineering objective. Meanwhile, there is now a growing argument to engineer natural materials systematically in the same way we have done for synthetics.

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