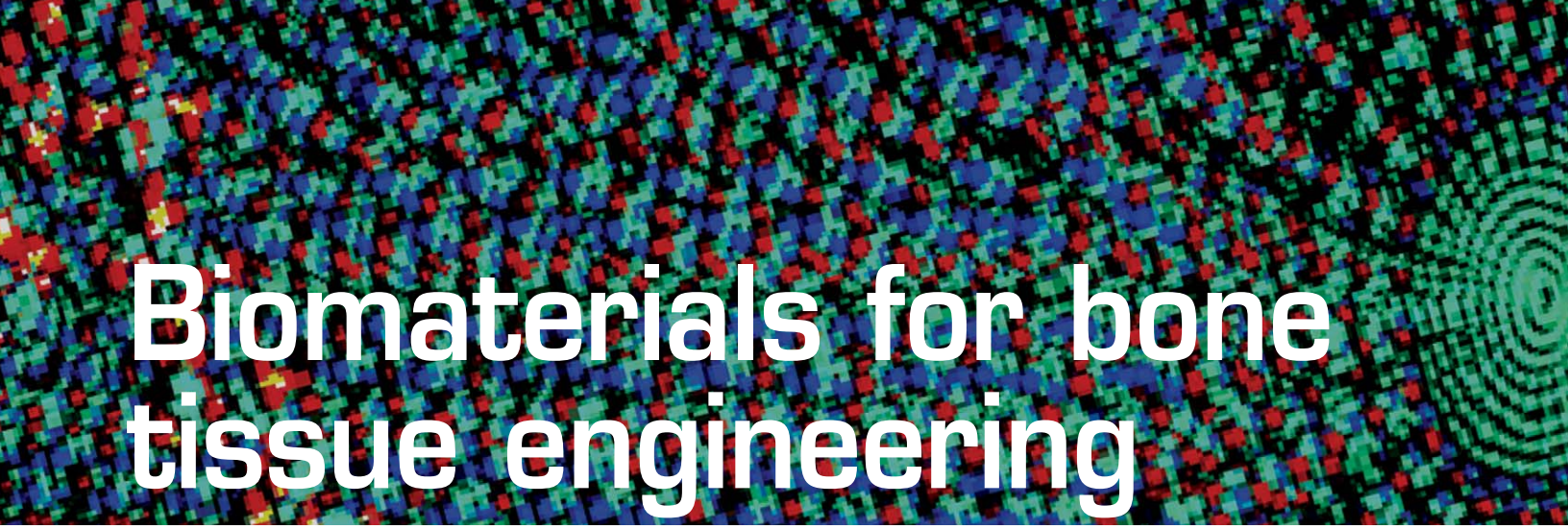

Biomaterials for bone tissue engineering

Materials that enhance bone regeneration have a wealth of potential clinical applications from the treatment of nonunion fractures to spinal fusion. The use of porous material scaffolds from bioceramic and polymer components to support bone cell and tissue growth is a longstanding area of interest. Current challenges include the engineering of materials that can match both the mechanical and biological context of real bone tissue matrix and support the vascularization of large tissue constructs. Scaffolds with new levels of biofunctionality that attempt to recreate nanoscale topographical and biofactor cues from the extracellular environment are emerging as interesting candidate biomimetic materials.

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Bone is a dynamic and highly vascularized tissue that continues to remodel throughout the lifetime of an individual. It plays an integral role in locomotion, ensures the skeleton has adequate load-bearing capacity, and acts as a protective casing for the delicate internal organs of the body. In addition to these structural functions, bone is intimately involved in homeostasis through its storage of Ca and P ions and by regulating the concentration of key electrolytes in the blood.

Clinical need for bone regeneration

The high regenerative capacity of bone, particularly in younger people, means that the majority of fractures will heal well without the need for major intervention. Despite this, large bone defects, as observed after bone tumor resections and severe nonunion fractures, lack the template for an orchestrated regeneration and require surgical intervention. Currently, the gold standard treatment is the use of a procedure called autografting, which involves the harvest of 'donor' bone from a non-load-bearing site in the patient (typically an easily accessible site like the iliac crest) and transplantation into the defect site[1]. Spinal fusion procedures also represent a growing need for massive autologous bone grafting, which have risen from being the 41st most common in-patient procedure in the US in 1997 to the 19th in 2003[2–4].

Transplanting autologous bone (i.e. bone from the patient) has the best clinical outcome as it integrates reliably with host bone and lacks the immune- and disease-related complications of allogeneic bone (i.e. bone from a human cadaver) or xenogeneic bone (i.e. bone from an animal source). Nevertheless, its use is severely hampered by its short supply and the considerable donor site morbidity associated with the harvest[5,6]. The search for new bone regeneration strategies is therefore

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a key international priority fueled by the debilitating pain associated with bone damage, and the increasing medical and socioeconomic challenge of our aging population – so much so that we are in the middle of a World Health Organization and United Nations Bone and Joint Decade global initiative!

Bone structure and properties

Distinct loading conditions influence the development of macroscopically diverse bony structures *in vivo* with carefully tailored shapes, mechanical properties, and spatial distributions. More than 206 different bones make up the skeleton, ranging from the long bones found in our limbs, short bones in the wrist and ankle, and flat bones in the sternum and skull, to irregular bones such as the pelvis and vertebrae. Bone tissue itself is arranged either in a compact pattern (cortical bone) or a trabecular pattern (cancellous bone)[7].

As with all organs in the body, bone tissue has a hierarchical organization over length scales that span several orders of magnitude from the macro- (centimeter) scale to the nanostructured (extracellular matrix or ECM) components (Fig. 1). Bone ECM comprises both a nonmineralized organic component (predominantly type-1 collagen) and a mineralized inorganic component (composed of 4 nm thick plate-like carbonated apatite mineralites)[8]. In addition, over 200 different types of noncollagenous matrix proteins (glycoproteins, proteoglycans, and sialoproteins) contribute to the abundance of signals in the immediate extracellular environment. The nanocomposite structure (tough and flexible collagen fibers reinforced by hydroxyapatite, HA, crystals) is integral to the requisite compressive strength and high fracture toughness of bone.

Biomaterials for bone repair

Not surprisingly given the pressing clinical need, the market for biomaterials-based treatments in orthopedics is growing at a rapid rate. While materials intended for implantation were in the past designed to be 'bio-inert', materials scientists have now shifted toward the design of deliberately 'bioactive' materials that integrate with biological molecules or cells and regenerate tissues[9,10]. In the case of bone, materials should preferably be both *osteoinductive* (capable of promoting the differentiation of progenitor cells down an osteoblastic lineage), *osteoconductive* (support bone growth and encourage the ingrowth of surrounding bone), and capable of *osseointegration* (integrate into surrounding bone).

Many bone substitute materials intended to replace the need for autologous or allogeneic bone have been evaluated over the last two decades. In general, they consist of either bioactive ceramics, bioactive glasses, biological or synthetic polymers, and composites of these[10–12]. The ideal basic premise, if following the tissue engineering paradigm, is that the materials will be resorbed and replaced over time by, and in tune with, the body's own newly regenerated biological tissue[9].

Bioactive inorganic materials

A wide range of bioactive inorganic materials similar in composition to the mineral phase of bone are of clinical interest, e.g. tricalcium phosphate, HA, bioactive glasses, and their combinations (Fig. 2)[10,13]. Bioactive glasses (Ca- and possibly P-containing silica glasses), for example, when immersed in biological fluid, can rapidly produce a bioactive hydroxycarbonated apatite layer that can bond to biological tissue. Furthermore, they can be tailored to deliver ions such as Si at levels capable of activating complex gene transduction pathways, leading to enhanced cell differentiation and osteogenesis[10,14,15]. The resorption rate of bioactive glasses and bioceramics can be tailored with crystalline HA persisting for years following implantation, while other calcium phosphates have a greater capacity to be resorbed but less strength for sustaining load[16]. The brittle nature of bioactive inorganic materials means that their fracture toughness cannot match that of bone and on their own are not good for load-bearing applications.

Polymers

Biological polymers, such as collagen and hyaluronic acid, are interesting candidates for tissue engineering and provide innate

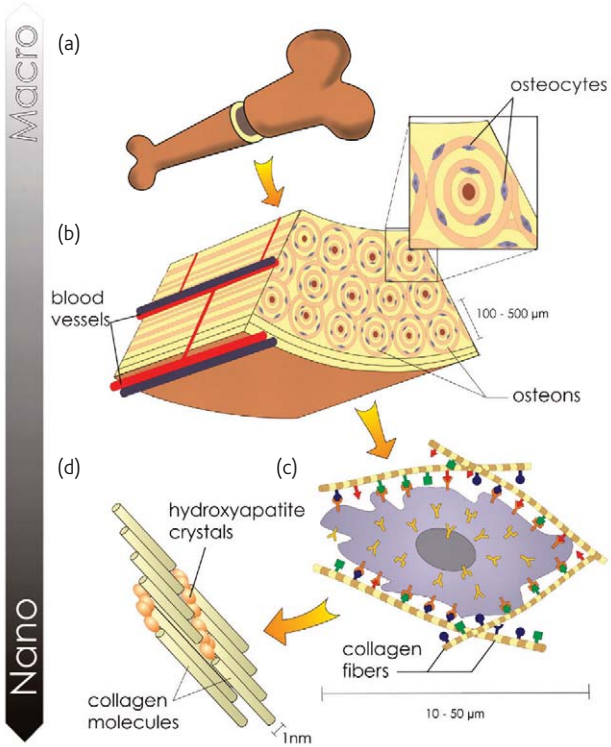

Fig. 1 Hierarchical organization of bone over different length scales. Bone has a strong calcified outer compact layer (a), which comprises many cylindrical Haversian systems, or osteons (b). The resident cells are coated in a forest of cell membrane receptors that respond to specific binding sites (c) and the well-defined nanoarchitecture of the surrounding extracellular matrix (d). (Reproduced with permission from[56]. © 2005 American Society for the Advancement of Science.)

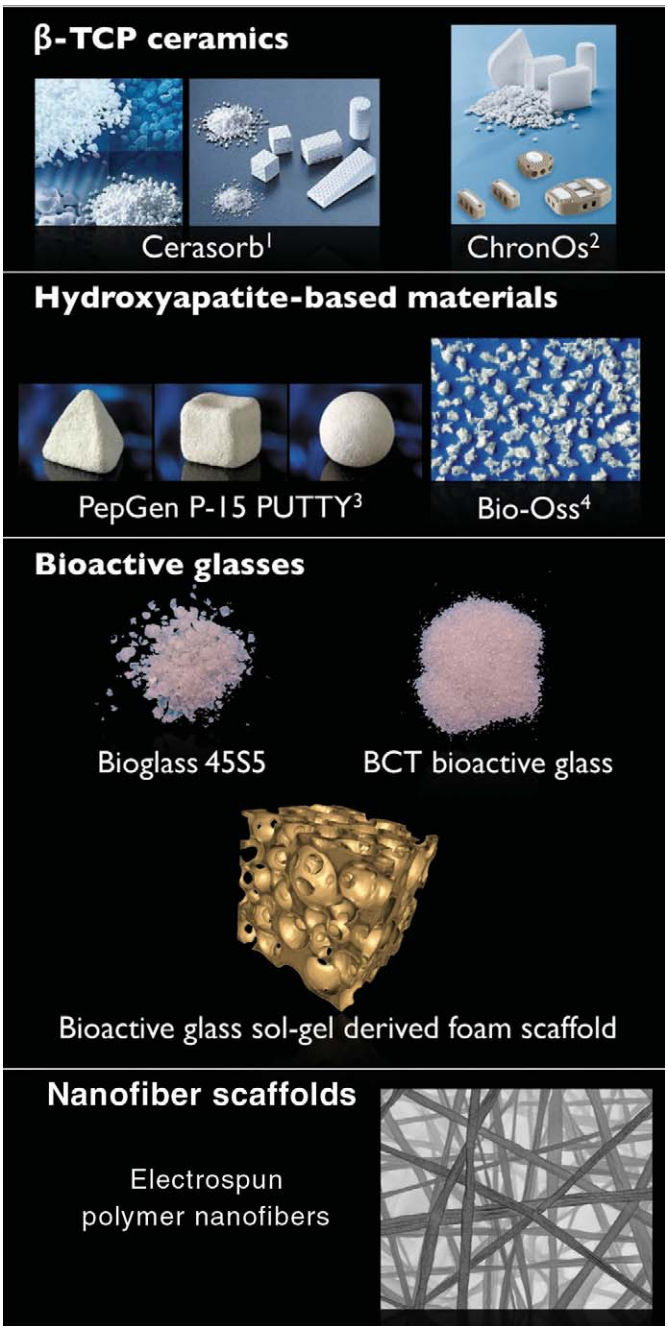

Fig. 2. Macromorphology of some examples of different bone graft materials. (Reproduced with permission from [1]Curasan AG, [2]Synthes, [4]Geistlich. [3]Courtesy of Dentsply Tulsa Dental Specialties. © PepGen® P-15.)

biological informational guidance to cells that favors cell attachment and promotes chemotactic responses. However, concern exists over immunogenicity, the potential risk of disease transmission, sourcing and poor handling, and weak mechanical properties.

Synthetic polymers such as polyfumarates, polylactic acid (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA (PLGA), and polycaprolactone offer a versatile alternative. They can be processed using techniques such as porogen leaching[17], gas foaming[17], phase separation[18,19], fiber meshing[20], supercritical fluid processing[21], microsphere sintering, and three-dimensional printing[22] to generate a range of three-dimensional scaffolds with different porosities and surface characteristics. Control over both global scaffold shape and three-dimensional microarchitecture is also benefiting from advances in solid free form fabrication (SFF). SFF includes a number of layer-by-layer manufacturing processes that enable complex three-dimensional anatomic scaffold architectures to be built using computer-aided design techniques and data from patient scans[23–27].

Most research focuses on polymers already used in devices approved by the US Food and Drug Administration (FDA), but high-throughput screening approaches to evaluate directly the effect of large libraries of novel polymers on cell phenotype are also underway[28]. Hydrogels (e.g. polyethylene glycol, alginate-based) are also popular as they can often be delivered in a minimally invasive manner and gelled *in situ* (e.g. photocrosslinked or ionically) to provide a three-dimensional cellular microenvironment with high water content. Their viscoelastic material properties seem particularly suitable for cartilage regeneration, although many applications in bone have also been explored[3,29–32]. Hydrogels have the advantage that chemical biofunctionalization and cell encapsulation and delivery are relatively straightforward[33,34].

Composite materials

Inorganic-organic composites aiming to 'mimic' the composite nature of real bone combine the toughness of a polymer phase with the compressive strength of an inorganic one to generate bioactive materials with improved mechanical properties and degradation profiles. For such composites, the alkalinity of the inorganic filler neutralizes acidic autocatalytic degradation of polymers such as PLA[35,36]. There is a growing recognition that a nanosized inorganic component is likely to be more bioactive than a micro-sized one. Tissue-engineered HA-collagen nanocomposite systems, for example, are emerging rapidly and showing promise[37].

Sol-gel processing is another interesting route that can combine inorganic/organic components at the nanoscale (e.g. creating a network from synthetic or biological polymers and inorganic silica chains)[38]. Recreating the same degree of nanoscale order in the organization of the mineral and organic components as found *in vivo*, however, is challenging. Mechanical properties of current composites still fall short of that of bone (nor do they attempt to match its anisotropy).

Bone tissue engineering

The synergistic combination of biomaterials and cell therapy is of great interest. Indeed, the potential of mesenchymal stem cells in the regeneration of bone has been highlighted since the 1980s[39]. Bone tissue engineering using biomaterials and cells ranging from primary adult osteoblasts (bone cells) to bone marrow mesenchymal stem cells has found a number of successes in animal models. However, the

majority of studies are in rodents and only a handful report orthoptic applications (i.e. in a bone defect) in larger animals[40–44]. Despite a few clinical successes, translation to human use has suffered from the poor predictive capacity for clinical outcome of the ectopic model in rodents[43,45–49]. This is understandable given the much smaller size of defects in rodents, higher bone remodeling rates, and lack of vascular supply in larger human defects, which is likely to result in significant cell death immediately after implantation of a cell-seeded biomaterial. It is worth considering that *in vivo* cells in metabolically active tissue are within 100 μm of a high oxygen source.

Vascularization of clinically relevant sized tissue engineering constructs remains both a limit in the transfer of tissue engineering from *in vitro* to *in vivo* and in transfer from animal to human systems. Introducing well-controlled, highly interconnected porosity into material scaffolds can aid subsequent permeability and the diffusion of oxygen and nutrients, as well as the creation of a three-dimensional vascular network. Other strategies for 'prevascularization' *in vitro* are emerging. For example, three-dimensional multiculture systems comprising progenitor cells, differentiated mature cells, and endothelial cells (cells that line blood vessels) can generate organized endothelial vessel networks throughout engineered tissue constructs, as recently demonstrated for engineered muscle implants[50]. Exogenous administration of potent angiogenic factors such as vascular endothelial growth factor (VEGF) can also stimulate vessel growth. However, application of such potent biologicals and indeed other growth factors involved in osteogenesis, such as bone morphogenetic proteins (e.g. BMP2 and BMP7, which are now in clinical use)[2,51–53], is not without drawbacks. Exogenous administration of BMPs is costly and runs the risk of causing heterotopic ossification (i.e. formation of bone outside the skeleton). Various growth factor delivery strategies may help overcome currently suboptimal release kinetics and the need for unphysiologically high concentrations of growth factors and their problematic short half-life (Fig. 3). Gene therapy approaches offer the possibility of local sustained gene expression from genetically modified cells, but the delivery vectors still need optimization[54,55]. It is worth noting that both of these approaches fail to recapitulate the complex temporal sequence and combination of growth factors involved in safe and stable bone formation *in vivo*.

Enhancing material biofunctionality

Incorporation of appropriate osteoinductive cues into scaffolds so that they can attract the patients' own stem cells post-implantation could obviate the need for cell delivery and exogenous growth factors. For this, more biomimetic environments must be created.

Cells are inherently sensitive to their surroundings. Topographic reaction (i.e. reaction to the surface landscape) of cells to grooves, ridges, wells, and other features at the micron scale and, more recently, the nanoscale is now well established[56]. Ongoing studies are showing effects on cell behavior ranging from changes in cell adhesion to modulation of the intracellular signaling pathways that regulate transcriptional activity and gene expression[57]. An interesting recent study of relevance to bone tissue engineering explored the

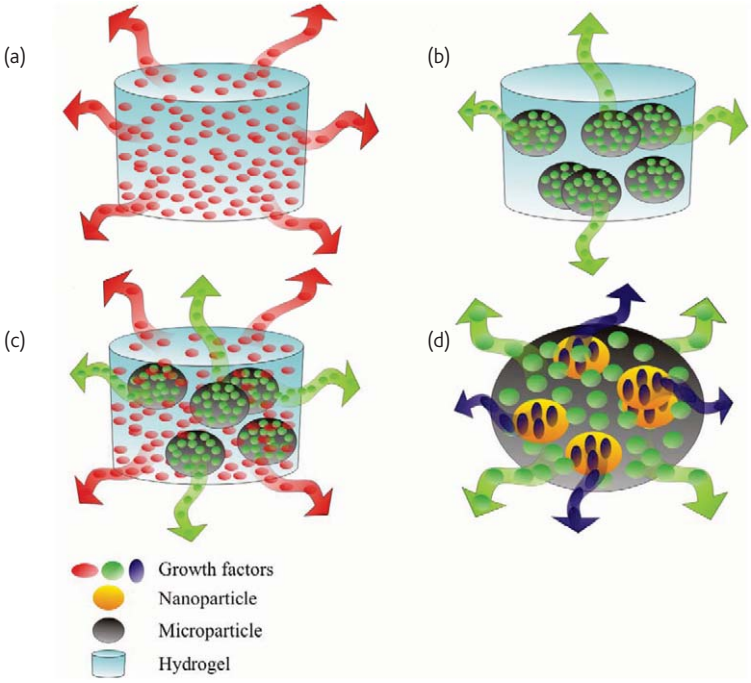

Fig. 3 Schematic of various potential drug delivery approaches for the delivery of (a, b) a single growth factor or (c, d) multiple growth factors. (Adapted from[94].) Other strategies to sequester and deliver growth factors are also under development such as the incorporation of growth factor binding peptides, proteins, and glycosaminoglycans into tissue scaffolds.

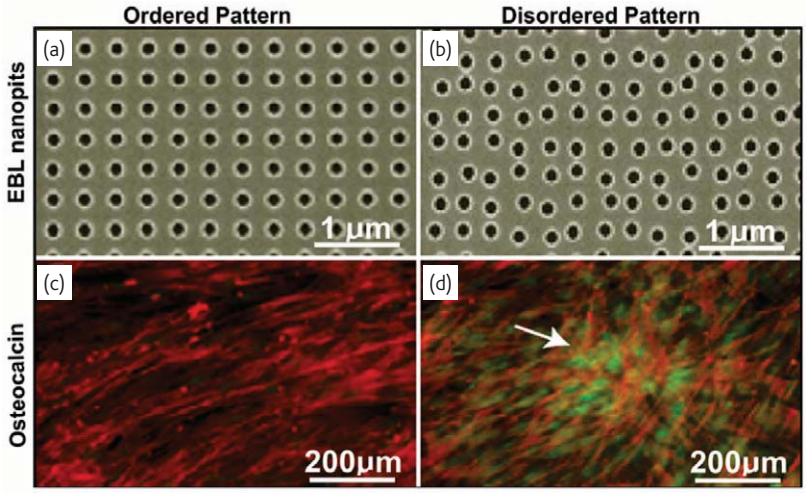

Fig. 4 Exploring the effect of different nanotopographies on cell differentiation. (a, b) Nanotopographies of increasing disorder were fabricated by electron beam lithography (EBL). The pits (120 nm in diameter and 100 nm deep) were generated (a) in a square arrangement and (b) with increasing disorder (displaced square ±50 nm from true center). The nanoscale disorder stimulates human mesenchymal stem cells to increase the expression of the bone-specific ECM protein osteopontin (d, arrow) compared with the ordered structure (c). (Reproduced and adapted with permission from [58]. © 2007 Nature Publishing Group.)

effect of random versus highly organized nanotopographical features, and found that the differentiation of mesenchymal stem cells to produce bone mineral is favored if there is a level of disorder in the presentation of nanoscale pits (Fig. 4)[58]. The relationship between nanoscale topographic features and protein adhesion and cell behavior is complex and remains to be elucidated in full, varying according to the shape and size of the topographic feature, as well as the protein and cell type.

Traditionally, materials design, while considerate of bulk tissue properties, has not encompassed the entire spectrum of biological length scale topography known to influence cell behavior (ranging from 10 nm to 100 μm). Nanophase reinforcements (such as HA-collagen nanocomposites or carbon nanotube polymer nanocomposites) are already generating improvements in bioactivity and mechanical properties such as flexural and compressive moduli[59–63].

Another approach to generate a biomimetically enhanced environment is to recreate the topographical context of native ECM through engineered three-dimensional nanofibrous matrices. The well-established, polymer-based processing methods of electrospinning and thermally induced phase separation, and protein self-assembly are all used to generate nanofibrous matrices[64,65].

There are now numerous examples of biological and synthetic polymer electrospun three-dimensional nanofiber matrices with high spatial interconnectivity, high porosity, and controlled alignment to direct cell orientation and migration[66]. These scaffolds may even be directly mineralized by introducing P-containing anionic functional groups into the backbone of the polymers or as pendant groups to induce the nucleation and deposition of HA[67,68].

Thermally induced phase separation, involving the thermodynamic demixing of a polymer solution into polymer-rich and polymer-poor phases, can also produce scaffolds with nanofibrous walls[69,70]. Not only is the surface area presented by the nanofibrous scaffolds greater, and hence the reactivity for proteins, there is also evidence that the types of proteins preferentially absorbed include those directly relevant to cell binding (such as fibronectin, laminin, vitronectin, and collagen)[69].

Self-assembled peptide or peptide amphiphile based systems, which take principles from protein folding and protein-protein interactions, can also be used to create well-ordered nanofibrous networks[71–77]. In the simplest case, even di- and tripeptides with hydrophobic end groups can self-assemble to form nanofibers[78,79]. Future strategies to improve the mechanical properties of peptide-based materials are necessary, if they are to be applied for load-bearing bone applications.

In addition to providing a backbone for favorable protein adsorption, the bioactive chemical and physical fine tuning of peptide-based or synthetic polymer systems, with additional cues for tissue development, is well underway. The modification of biomaterials can take on different levels of complexity, from relatively simple changes in the hydrophilicity of the material to functionalization with charged groups, peptides, or full proteins (Fig. 5).

The incorporation of bioactive peptide motifs such as arginine-glycine-aspartic acid (RGD), which is recognized by the cell's transmembrane integrin receptors, is perhaps the most commonly adopted strategy to enhance functionality[78]. The cell response is always specific to particular ligand surface densities and binding affinities, is often biphasic (e.g. migratory response), and is modulated by co-localization with synergistic ligands[80–84]. Incorporating proteolytically degradable peptide motifs, such as those recognized by cell-secreted matrix metalloproteases, is now a popular route to biodegradability that is more in tune with tissue remodeling and regeneration[31,72].

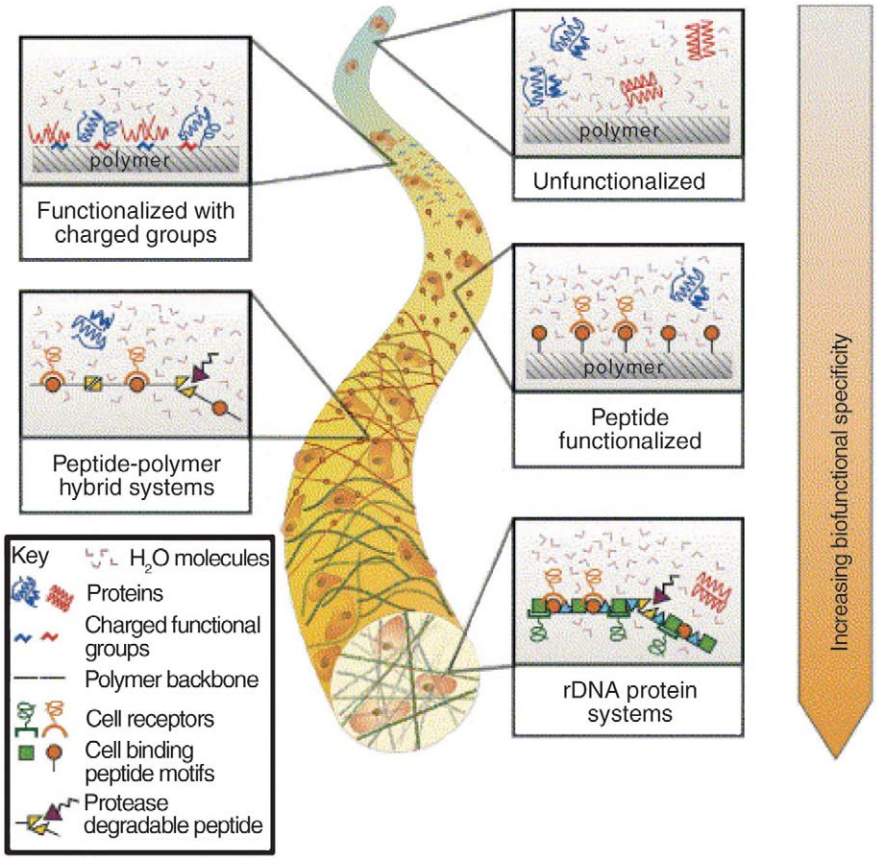

Fig. 5 Enhancing material biofunctionality. Control over cellular interaction for bone and cartilage repair can be achieved through scaffold material design. Several different examples are presented here, ordered by increasing biofunctional specificity. Unfunctionalized: unmodified polymer surfaces nonspecifically absorb proteins through weak interactions between the protein-water and water-surface interfaces. Functionalized with charged groups: chemical modification of the polymer surface with different charged end-groups (e.g. –OH–, –COO–, $–NH_3^+$) increases electrostatic interactions and may lead to stronger protein absorption and structural rearrangements, which may expose hidden binding sites for cell attachment. Peptide functionalized: the incorporation of peptide motifs (e.g. RGD) can be used to increase the binding of specific cell receptors, directing cell behavior. Peptide-polymer hybrid systems: by including peptides, such as protease-sensitive degradation sites, within the polymer backbone, the scaffolds can be further enhanced to permit cell-mediated migration and degradation. rDNA protein systems: synthetic artificial proteins can be designed to structurally and functionally resemble specific biological ECM constituents using recombinant DNA (rDNA) technology. (Reproduced with permission from[95]. © 2006 Elsevier Ltd.)

More recently, the incorporation of other biological components from the ECM such as glycosaminoglycans (GAGs) and, in particular, heparin is yielding interesting results[85–87]. Heparin, once incorporated into the fabric of the biomaterial, can be recognized by heparin-binding domains found in proteins relevant to cell attachment (e.g. fibronectin and vitronectin), cell proliferation (e.g. basic fibroblast growth factor), osteogenic cell differentiation (e.g. bone morphogenetic proteins among others, pleiotrophin), and thus used for their controlled sequestering and delivery. Heparin has been incorporated, for example, as heparin-functionalized poly(ethylene glycol) (PEG) hydrogels[85], by electrospinning of a heparin-PEG star copolymer into PLA fibers[86], or used to nucleate the self-assembly of nanostructures from designed peptide amphiphile molecules[88]. In this latter example, relatively rigid nanofibers are generated that can be loaded with angiogenic growth factors including VEGF, which when implanted *in vivo* stimulate much greater angiogenesis than the delivery of growth factor alone (Fig. 6).

All these developments are likely to enable even closer matching of scaffolds to the *in vivo* environment. In addition, there have been notable successes in bone engineering by maximizing the *in vivo* environment as its own bioreactor for *de novo* tissue regeneration[3,89]. In our recent study, controlled *in vivo* bioreactor environments were created between the tibia and the periosteum, a mesenchymal layer rich in pluripotent cells, to induce the body's natural healing mechanisms to generate new tissue and provide all the necessary cells and factors in the correct temporal and biochemical sequence (Fig. 7)[3]. Volume is given to the artificial bioreactor space by minimally invasive delivery of a Ca-rich gel that supports massive bone ingrowth and, importantly, generates bone with the correct hierarchical organization, anisotropy, and mechanical properties to match that of native bone. The importance of a highly controlled environment is highlighted by the ability to generate cartilage exclusively in the bioreactor by inhibiting angiogenesis and promoting a more hypoxic environment.

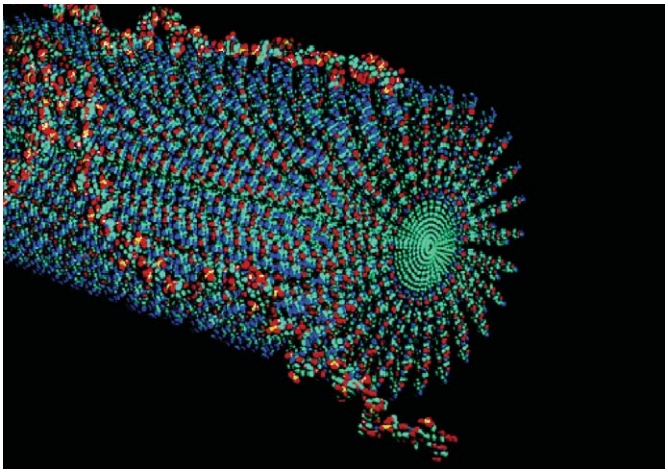

Fig. 6 Schematic representation of a heparin-nucleated nanofiber designed to promote the growth of blood vessels. The cylindrical nanostructure is formed by the aggregation of positively charged peptide amphiphile molecules. The peptide amphiphile molecules have the capacity to bind to the negatively charged heparin chains, and the polyion nucleates the fiber. (Reproduced with permission from[88]. © 2006 American Chemical Society.)

Challenges and future directions

The field of bone tissue engineering is at an exciting point, with enormous research activity focused on delivering new and improved biomimetic materials. The level of biological complexity that needs to be recapitulated within a synthetic three-dimensional environment is still uncertain. Further elucidation of the communication between cells and of the complex interplay between cells and their matrix will help focus strategies to enable the presentation of biofactors in the correct context both chemically, temporally, and in terms of their distribution. Similarly, the clinical application of surface structuring approaches will require further understanding of the interactions occurring at the cell surface/substrate interface. Vascularization of large tissue constructs remains a significant challenge and some engineering-based approaches to try and overcome this have been discussed here. It is worth noting that advances in microsurgical techniques are also underway to allow reconstructive surgeons to generate so-called 'axially vascularized' tissues that can overcome some of the existing problems in achieving rapid vascularization of implanted biomaterials[90]. This highlights the importance of close interaction between the surgical and cell biology communities as we move from the bench closer to the bedside. The harvest of pluripotent mesenchymal cells from sources other than bone marrow, for example from the periosteum or adipose tissue, also warrants consideration[30,91].

Advances in materials processing are also having a positive impact on the field. In the body, bone often has a structurally important interface with other tissues such as cartilage and ligament/tendon, for which designed scaffolds can be used to create tissue interfaces. For example, computer-aided design and SFF polymer/ceramic composites have been used to create a construct for a bone-cartilage interface by seeding chondrocytes (cartilage cells) within the cartilage portion and BMP-7 transduced cells on the ceramic portion[92]. The potential to combine three-dimensional printing of scaffolds with three-dimensional printing of cells and biologics, while currently challenging, will enable the development of new designer material/biofactor hybrids[23,93]. Soft material routes like sol-gel processing might also be a strategy to incorporate biomolecules during scaffold fabrication, although this is still under development. It is likely that biofunctionalization strategies

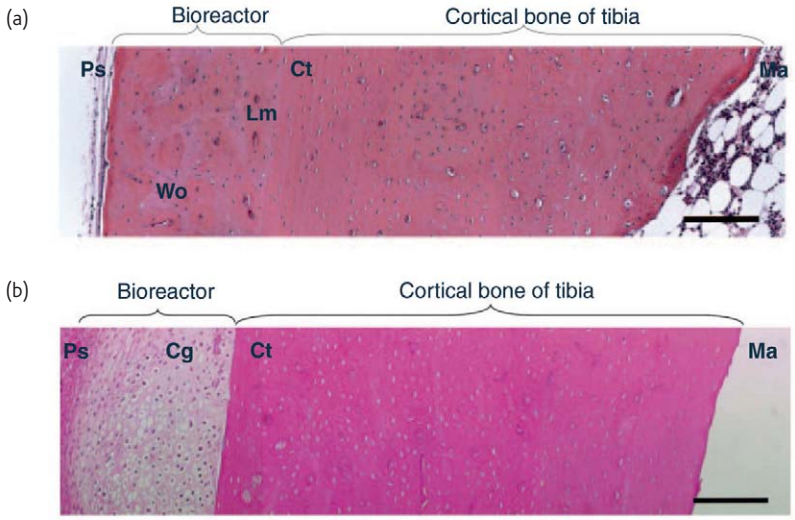

Fig. 7 Histological characterization of the neo-tissue produced within the in vivo bioreactor in absence of growth factors. (a) In vivo bioreactor for bone: hematoxylin and eosin stained cross section of the bone bioreactor, adjacent cortical bone, and marrow cavity six weeks after a Ca-rich alginate gel was introduced into the bioreactor. (b) In vivo bioreactor for cartilage: hematoxylin and eosin stained cross section of cartilage in the bioreactor and adjacent cortical bone ten days after hyaluronic acid based gel containing Suramin was introduced into the bioreactor. Ps, periosteum; Wo, woven bone; Lm, lamellar bone; Ct, cortical bone; Cg, cartilage; Ma, marrow. Scale bar = 300 μm. (Adapted with permission from[3]. © 2005 National Academy of Sciences.)

will continue to receive a well-deserved focus, as will approaches to better integrate micron- and nanoscale features into designed scaffolds. Developments in this field will find a wealth of applications in our aging population. mt

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