Post-print of: Serás-Franzoso, J. , et al. “Integrating mechanical and biological control of cell proliferation through bioinspired multieffector materials” in Nanomedicine (Ed. Future medicine), 2015, Vol. 10, No. 5, p. 873-891. The final versión is available at DOI 10.2217/nnm.15.5

Integrating mechanical and biological control of cell proliferation through bioinspired multi-effector materials

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Abstract

In nature, cells respond to complex mechanical and biological stimuli whose understanding is required for tissue construction in regenerative medicine. However, the full replication of such bimodal effector networks is far to be reached. Engineering substrate roughness and architecture allows regulating cell adhesion, positioning, proliferation, differentiation and survival, and the external supply of soluble protein factors (mainly growth factors and hormones) has been long applied to promote growth and differentiation. Further, bio-inspired scaffolds are progressively engineered as reservoirs for the *in situ* sustained release of soluble protein factors from functional topographies. We review here how research progresses towards the design of integrative, holistic scaffold platforms based on the exploration of individual mechanical and biological effectors and their further combination.

Keywords

Topography; Regenerative medicine; Adhesion proteins; Hydrogels; Growth factor release; Bioscaffolds

Introduction

A deep understanding of cell adhesion, positioning, migration, proliferation, apoptosis and differentiation and the precise control of the underlying mechanisms are necessary for the generation of fully functional artificial tissues. The comprehension of how cells organize into complex structures requires the identification of the different participating effectors and how they combine for specific, time-dependent cell responses. This is especially critical for the reconstruction of damaged tissues that involves the controlled cultivation of stem cells on artificial scaffolds, either straightforward *in vivo*, or *ex vivo* followed by implantation into damaged organs. In both cases, manmade scaffolds are expected to be biocompatible, mechanically stable and when required fully biodegradable (depending on how the regenerative process has been designed). They must also provide a bio-inspired topography within cell dimensions range to support cell colonization, mimicking to that offered *in vivo* by the extracellular matrix (ECM). This is because mechanic stimuli have been revealed as critical for cell proliferation, positioning and differentiation, acting through cell sensing and mechanotransduction events [(1)]. Then, the *de novo* designed scaffolds for tissue engineering must address precise topographical requests at micro and nano scales apart from exhibiting defined material properties affecting cell behavior such as two-dimensional/three-dimensional (2D/3D) geometry, appropriate stiffness and surface charge.

In the ECM, mechanical stimulation is combined with the activity of biological effectors mainly based on soluble molecules such as hormones, growth factors (GF), signal transducers and probably a set of still unidentified agents released by neighboring cells. Also, the ECM itself, based on diverse types of protein materials, displays cell adhesive and topographical properties that regulate cell fate [(2)]. The combined action of biological and mechanical agents generates a complex stimuli pattern that supports the dynamics of tissue formation and vascularization [(3)]. Then, any potential of a synthetic scaffold to act, in addition to topographic modulator, as a reservoir of bioactive compounds for their sustained release is highly appealing, especially for *in vivo* applications in which external drug supply might be restricted. In this regard, mechanical stimulation in combination with the supply of these factors represents the best approach to mimic the natural cell environment in artificial sets. However, the enormous complexity of the natural effector network and the synergistic activities of their components delay the desirable combined application of modulators in tissue engineering. Then, both type of effectors are often developed and tested separately, and only a moderate number of strategies are addressing the integrative supply of mechanical and biological signals. Importantly, proteins represent particularly intriguing materials as they can provide, simultaneously, architecture and functionality to cell substrates. The main trends in the topographical design of scaffold materials as well as the biological nature of protein-based effectors of relevance in tissue engineering are revised here. We particularly stress emerging developmental routes towards biofunctional scaffolds empowered to present both mechanical and biological stimuli in a cell sensing range.

Engineering scaffold topography

Plain topographical stimuli. Synthetic topographies affect basic functions in almost all types of mammalian cells. Therefore, engineering substrates within the size range at which ECM mechanical effectors trigger cell responses [4] offers a powerful tool to study and regulate complex cell functions such as adhesion, migration, cytoskeleton reorganization and cell polarization [5,1] that might be useful and exploitable for specific tissue engineering purposes. Although responses vary across cell type and substrate properties, some general lessons can be extracted from the rapidly growing body of literature (Table 1). These data could then be exploited to iteratively probe, engineer and improve cell–nanotopography interactions for tissue engineering applications though the manipulation of the mechanical stimuli to which cells are exposed [6].

Mechanical stimulation has been explored through the lithographical modification of polymers or other surfaces (*top-down* approach) to generate micro- and nano-grooves or pits (*bottom-up* approach) [7,8]. The use of such modified substrates permits, in addition, the regulation of the expression of cell adhesion molecules [9], the distribution of focal adhesions [10] and the orientation of whole cells as well as their morphological appearance [11]. Microcontact printing (μCP)[12] is recognized as a cost-effective, fast and versatile technique to control surface chemistry at the microscale over considerably large areas (up to hundreds of mm^2). The range of materials that can be used to cover surfaces using this method is broad [13]: self-assembled monolayers (SAM´s) [14], proteins [15] and nucleic acids [12] among others giving rise to functional surfaces [16] which are obtained by multistep protocols. Although in most cases the "ink" used in this printing procedure consists of a solution of the molecules of interest, such soft-lithographic method can also be extended to pattern colloidal particles [17] or even bacterial cells [18], expanding the functionalities of the engineered surface.

Alternatively, several categories of particulate materials have been explored for the nanomorphological modification of scaffold surfaces (*bottom-up* approach), including ceramics, polymers and nanotubes [19,20,21,22]. Particle-based surface decoration is highly promising since it is less dependent on the chemical nature of the scaffold material in contrast to lithographical modification. It allows, in some materials, important levels of topographical flexibility and controllable effects on cells, as exemplified by the use of nanotubes as substrate modifiers. In this context, the viability and activity of MSCs cultured on TiO_2 nanotubes can be controlled by the tube diameter [23]. Vertically aligned TiO_2 nanotubes with a diameter larger than 50 nm dramatically reduced cell activity and caused programmed cell apoptosis. Compared to smooth TiO_2 surfaces, a lateral spacing of 15–30 nm strongly promoted focal contact formation and enhanced cell activities [24]. Using this platform, the influence of integrated nanoscaled topography and GFs to stem-cell fate has also been investigated, facilitating the further developments of medical implants and materials.

Combined topographical stimuli. In this context, recent approaches focus on two or more combined engineering strategies to achieve complex combined stimuli. Hot embossing has been applied to control topography and μCP for a chemical patterning, to obtain substrates with grooves covered with perpendicular stripes of proteins [25], while a similar architecture has been also generated but with parallel patterns [26]. Recknor and coauthors co-cultured

astrocytes with adult rat hippocampal progenitor cells over chemically modified micropatterned polystyrene substrates and they preferentially acquire neuronal morphology depending on the microstructuration of the substrate [27]. These examples indicated that substrate topography in synergy with chemical modification and biological guidance facilitates cell differentiation. Also in this regard, we have recently shown that bacterial inclusion bodies (IBs), pseudospherical protein clusters spontaneously formed in recombinant bacteria, can be used as biocompatible materials for surface decoration and stimulation of mammalian cell spread. Since IB formation is multigenetically determined through the cell quality control system, mechanical, morphological, structural and biological properties of IBs can be adjusted by the genetic manipulation of the producing cells. IBs show a positive impact on colonization and proliferation [28,29], and being highly bioadhesive materials, cell expansion on IB-decorated surfaces has been proven to be synergistically supported by both favored adhesion and mechanical stimulation of cell division [30]. In micropatterned surfaces, cells preferentially adhere to IB-rich areas, aligning and elongating according to the IB pattern and choosing the shortest way to reach new adhesion spots on the IBs [31]. Such 2D engineering technique fills the gap between existing techniques which are based on the local modification of the chemical nature of the surface and those based on the modification of the topography at the nanoscale level by physical methods because IBs combine at the same time biofunctionalization and topographical modification of the roughness, as discussed in more detail above.

3D topographies. 3D scaffolds are expected to mimic the ECM and the natural cell environment more accurately than conventional 2D surfaces. Apart from metals, ceramics, protein-based hydrogels and carbon nanotubes, a spectrum of biocompatible and biodegradable polymers is being explored for *ex vivo* 3D culture and subsequent implantation, including hyaluronic acid (HA), polylactic acid (PLA), polyglycolic acid (PLGA), chitosan (CHT), hydroxyapatite, polycaprolactone (PCL), polyanhydrides, polyorthoesters and dendrimers [32,33,34]. Controlling the material architecture during biofabrication permits the pre-definition of porosity for mass transfer and proper colonization of the inner surfaces.

In addition, 3D scaffolds are expected to offer disordered mechanical stimuli for mechano-transduction events [35,36], required for a fine control of cell response, more efficiently than 2D substrates. Since ideally, mechanical stimulation should act synergistically with biological signals, 3D scaffolds might be appropriate as combined with sets of soluble factors embedded in the matrices, as discussed in deep below. This is exemplified by the emerging biomimetic materials used in implants for bone regeneration such as nano-hydroxyapatite/polyamide66 and derivatives [37] that show excellent biocompatibility, stability and osteoconductivity. When used in the fabrication of screws can be loaded with GFs to confer additional biological activities to the material and successfully fix intercondylar femur fractures [38]. In addition, related hydroxyapatite materials can be loaded with antibiotics for sustained release *in vivo* to prevent bacterial infections subsequent to surgery [39].

Table 1. Topographical control of cell proliferation, morphology and positioning, illustrated by representative examples.

			Feature geometry		Impact on cell	Reference
Substrate material	**Feature type**	**Cell type**	Width	Depth		
Quartz	Grooves	Murine P388D1 macrophage	0.5, 5, 10, 25 µm	0.5, 5 µm	Orientation change and Elongation, more in wider grooves	(40)
Quartz	Grooves	Mesenchymal stem cells	1. 4 µm	1.1 µm	Alignment better in the widest grooves	(41)
Quartz	Grooves	Fibroblasts	12.5 µm	5 µm	Gene expression largely changed	(42)
Quartz	Grooves	Murine macrophages	2-10 µm	30-280 nm	Higher phagocytotic activity when topography fiber	(43)
Quartz	Grooves	Human corneal ECs	1-4 µm	N/D	Elongation	(44)
Silicon	Grooves	Humancorneal epithelial cells	330-2100 nm	600 nm	Perpendicular alignment for 400–800 nm pitch. Parallel for 1600–4000 nm	(45)
Silicon dioxide	Grooves	Fibroblasts	0.5 µm	1 µm	Strong alignment	(46)
Silicon dioxide	Grooves	Keratinocytes	0.5 µm	1 µm	No alignment	(46)
PMMA	Grooves	BHK cells	2, 3, 6, 12 µm	0.2, 0.5, 1.1,1.9 µm	Alignment increased with depth and decreased with width	(47)
PMMA	Steps	BHK	1-18 µm	N/D	Alignment at steps	(47)
PMMA	Pillars	Fibroblasts	100 nm	160 nm	Smaller, less organized actin cytoskeleton	(48)
PCL,PMMA	Pits	Fibroblasts	35, 75,120 n m	N/D	Reduced adhesion, orientation and distinction of symmetries	(49,50,51)
PCL, PMMA	Nanopit	Human fibroblasts	35-120 nm	N/D	Adhesion decreased and biased orientation	(52)
PCL	Nanopit	Human fibroblasts	35-120 nm	N/D	Spreading decresed. Increased filopodia	(53)
PCL	Nanopit and nanopost	Rat fibroblasts	60-150 nm	N/D	Adhesion decreased and increased adhesion on random	(54)

					nanoposts	
PS	Grooves	Rat astrocytes	10 µm	3 µm	Less adhesion, strong alignment	[55]
PS	Grooves	Fibroblasts	20-1000 nm	5-530 nm	No alignment for depths <35 nm or widths <100 nm	[56]
PS	Grooves	Rat bone cells	1-10 µm	0.5-1.5 µm	Width > 5 µm: cells followed the surface. Narrow grooves: cells bridge	[57]
PS	Grooves	Rat bone marrow cells, osteoblasts MC3TC	Micro (manual)	N/D	RBMC influenced by grooves (osteoblast differentiation) MC3T4 not influenced	[58]
PS	Grooves	rC6 glioma	266 nm	N/D	Elongation	[59]
PS	Grooves	hEKCs (HEK-293)	200-430 nm	N/D	Elongation. Increased proliferation	[60]
PS	Grooves	Human corneal ECs	2-20 µm	N/D	Elongation, lower cell area.	[61]
PS	Grooves	Rat bone cells	1-10 µm	0.5-1.5 µm	Large grooves: focal adhesions all over the surface; Narrow grooves: only on the edges	[57]
PS	Nanopost	HeLA	160-1000 nm	N/D	Spreading decreased. No effect on proliferation	[62]
PS and PBrS	Random	Human endothelial	13, 35, 9 5 nm	N/D	Round cells on PS, Arcuate morphology largest for the 13 nm islands	[63]
Polyimide	Grooves	Osteoblasts	4 µm	5 µm	Strong alignment and elongation no changes in adhesion	[64]
PDLA	Grooves	Schwann cells (nerve cells)	10 µm	3 µm	Strong alignment	[65]
PLGA, PU, PCL	Random	Bladdersmooth Muscle cells	206, 370 nm	N/D	Enhanced adhesion	[66]
Epoxy	Grooves	Human fibroblasts	0.5 µm	1 µm	Cytoskeleton oriented with grooves	[67]
PLLA, PS	Grooves	Rat bone cells	1,2,5,10 µm	0.5,1,1.5 µm	Better mineralization with feature diameter of 1 µm and feature width of 1–2 µm	[68]
PDMS	Grooves	Human embryonic stem cells	600 nm	600 nm	Reduced proliferation	[69]
PDMS	Wells	Human fibroblasts	2,5,10 µm	N/D	With 2 and 5 µm better proliferation. 10 mm has no effect	[70]

PDMS	Grooves	Human endothelial cells	600 nm	N/D	Decreased proliferation. Elongation, lower cell area	[71]
PDMS	Grooves	Human embryonic stem cells	600 nm	N/D	Elongation. Adhesion decreased and decreased proliferation, cytoskeleton disrupting agents impact response	[69]
PDMS	Grooves	Human mesenchymal stem cells	350 nm-10 µm	N/D	Elongation, lower cell area. Decreased proliferation, differentiation into neuronal lineage	[72]
PMMA	Wells, random	Human mesenchymal stem cells	120 nm	100 nm	Stimulated differentiation and production of bone mineral in vitro	[36]
PMMA	Random	Bone marrow cells (stem cells)	100-2000 nm	N/D	Differentiation to osteoblasts promoted, cells organize into superstructures	[73]
PMMA	Nanopit	Human mesenchymal stem cells	300 nm	N/D	Osteogenic differentiation	[36]
PMMA	Pillars	Fibroblasts	100 nm	160 nm	Less spreading	[74]
PDMS, PMMA	Grooves	Bovine smooth muscle cells	350 nm	N/D	Elongation. Decreased proliferation, polarized microtubule organization center	[75]
PLGA	Random	Rat aortic smooth muscle cells, rat aortic endothelial cells	Nano-range	N/D	Improve on cell densities with the nanostructure	[76]
PEG	Nanopost	Rat cardiomyocytes	150 nm	N/D	Adhesion increased	[77]
PEG	Nanopost	mP19EC stem cells	300-500 nm	N/D	Adhesion increased	[78]
PC	Nanopit	Human bone marrow cells	300 nm	N/D	Spreading decresed. Constant filopodia formation N/D	[79]
PC	Nanopit	Human osteoblasts	300 nm	N/D	Adhesion decreased. Reduced area of adhesion complexes	[80]
PGS	Grooves	Bovine endothelial	2 µm	N/D	Elongation	[81]

		cells				
Si	Nanopost	Fibroblasts	50-600 nm	N/D	Spreading decresed. No effect on proliferation	[82]
Si	Grooves	PC12	70-1900 nm	N/D	Elongation. Cooperative neurite extension	[83]
Si	Nanopit	Human fibroblasts	80 nm	N/D	Spreading decresed	[79]
Si	Grooves	Human corneal ECs	70-2100 nm	N/D	Elongation, lower cell area. Adhesion increased. Biased lamellipodioa extension	[45,84]
Si	Grooves	Human fibroblasts	50-600 nm	N/D	Elongation, lower cell area. Decreased proliferation	[82]
Ti	Grooves	Fibroblasts	3-5 nm	N/D	Elongation. No effect, increase in fibronectin mRNA incorporation	[85]
Ti	Grooves	Rat endothelial cells	750 nm-10 µm	N/D	Increased adhesion and longation	[86]
Ti-coated Si	Grooves	T24, human bladder carcinoma	15 µm	200 nm	Nanopillars: less round and more stellate, smaller Grooves: elongatio	[87]
Alumina	Pillars, pores	Mouse marrow stromal cells	110, 72 nm	N/D	Proliferation increased 45% increased osteoblast differentiation	[88]
Silica on PEI-coated silicon gradient concentration	Beads	Rat calvarial osteoblasts	73 nm	73 nm	Particles (nanotopography) reduced cell proliferation	[89]

Abbreviationlist:N/D:non-determined;PMMA:poly(methylmethacrylate);PDMS: poly-dimethyl siloxane; PC: polycarbonate; PS: polystyrene; PLLA: poly(L-lactideacid); PET: poly(ethyleneterephthalate); PBrS: poly(4-bromostyrene); PCL: polycaprolactone; PDLA: poly(D,L-lactic acid); PLGA: polylactic-co-glycolic-acid; PU: polyether-urethane.

Tailoring bio-functional protein materials

Promoting cell adhesion through protein coating. Often, fabrication constraints, biocompatibility, durability and the need to control precise architectural, topographical or chemical profiles impose the use of unfriendly materials as prototype scaffolds for tissue engineering. Coating these scaffolds with ECM proteins such as fibronectin (FN), the most adhesive glycoprotein, collagen I or III, laminin-I, elastin and vitronectin facilitates cell adhesion and colonization (Table 2). These proteins adsorb to almost any surface, including metals,

organic biopolymers and ceramics [90], by binding forces responsible for the "Vroman effect" [91]. Due to their difficult extraction from natural sources, many ECM proteins have been produced in recombinant forms. Since during adsorption full-length proteins may suffer conformational changes that hide functional domains critical for cell interaction [92], coating with functional ECM protein fragments, like the 120-KDa FN segment or peptide epitopes like RGD (Arg-Gly-Asp), found in many adhesive ECM glycoproteins, might be highly convenient. RGD motives need to maintain its native stereo conformation (cyclic form) to exhibit full binding activity (240 times more active than the linear peptide) [93], what poses important challenges in peptide design and synthesis. Other integrin-binding peptides from ECM are REVD, KQAGDV and PHSRN derived from FN; YIGSR, IKLLI, LRE, LRGDN, PDGSR, LGTIPG and IKVAV derived from laminin or GFOGER p15 and DGEA derived from collagen. Scaffold coating with adhesive ECM epitopes gives the opportunity to control not only peptide density but also clustering through nanopatterning, which in turns regulates cytoskeletal organization, focal contact, proliferation, adhesion and differentiation [94], as discussed above.

Also, scaffolds themselves can be made of collagen, elastin or polysaccharide nanofibers like hialuronic acid (HA), cellulose, alginate, chitin and chitosan. Scaffolds of native collagen type I have been extensively used in cell biology and 3D collagen gels have been successfully applied to skin regeneration and cartilage repair, as they promote convenient cellular behavior in terms of migration, shape and differentiation. Scaffolds of native and recombinant elastin have been used *in vivo* for tissue engineering of skin and vascular tissues with promising results. On the other hand, HA scaffolds are widely employed in ophthalmology, as joint lubricants and in tissue engineering of cartilage and skin, due to the pleiotropic cellular effects derived from HA-CD44 interaction (for a review, see [95]). Finally, keratin biomaterials derived from human hair are suitable to induce cell adhesion, proliferation and migration [96].

In a related scenario, elastin-like polypeptides (ELPs) are recombinant alternatives derived from elastin, which maintain biocompatibility for *in vivo* applications and show structural responsiveness to temperature. ELPs are formed by repeats of the elastin primary sequence VPGXG. They are used to construct scaffolds for the regeneration of different tissues (dermal, vascular, cardiac, cartilage), but also to coat different materials too hydrophobic or negatively charged to allow cell adhesion and growth [97].

Unconventional and emerging adhesive proteins. Silk fibroin (SF), a silk protein produced by silkworms, exhibits excellent biocompatibility, good oxygen and water vapor permeability, biodegradability, triggering a minimal inflammatory reaction [98] . In practice, SF has been used in cosmetics, as a medical material in human medicine and as food additive. Electrospun SF matrices have been developed as a support for culture of fibroblasts and keratinocytes [99], bone marrow stem cells (BMSC) [100], human aortic endothelial cells (HAEC) and human coronary artery smooth muscle cells (HCASMC) [101], resulting in positive effects on cell adhesion, viability, growth, and differentiation. On the other hand, mussel adhesive proteins, with outstanding adhesive properties even in aqueous environment, have been used as scaffolds for bone regeneration [102] and have inspired the generation of polydopamine in different formats, useful in re-endothelialization of artificial vessels [103]. They are commercially available as Cell-Tak (BD Bioscience, Corning), which is an extracted mixture of fp-1 and fp-2,

useful to immobilize *in vitro* different cells and tissues on glass, plastic or even metals (Table 2).

Also, many non-natural peptides relevant to cell adhesion, proliferation and spreading have been obtained by genetic modification of natural sequences, by screening combinatorial peptide libraries and by combining bioinformatics and protein structural data to adjust and optimize adhesive properties for specific cell types. These strategies and the resulting peptides have been extensively revised and summarized elsewhere [(104)].

Cell adhesion in bone tissue engineering. Many studies addressed to elucidate the molecular basis of osteogenesis from MSCs have demonstrated that substrate protein coating might be decisive in different phases of bone generation, mimicking the activities of corresponding soluble protein versions. FN to promote initial MSC adhesion and proliferation, bone morphogenetic protein 2 (BMP-2) as active agent in a second stage of differentiation and bone matrix production, osteopontin (OPN) and bone sialoprotein (BSP) to promote cell adhesion and differentiation into osteoblasts and osteoclasts, and tenascin (TN) to induce mineralization and new bone formation. Interestingly, FN effects differ depending on the type of coated material, probably due to conformational changes induced upon adsorption. In fact, the adhesion properties of OPN and BSP are enhanced when coated in their oligomerized forms [(105)].

Developing functional drug-releasing biomaterials

Hydrogel architecture. As mentioned above, the recreation of micro- and nano- topographies and cell-friendly surfaces for efficient attachment should be combined with biochemical signaling, desirably achieved by the release of soluble factors such as GFs, cytokines and other bioactive molecules (Figure 1). Among the broad range of available materials, the supramolecular organization of fibrous structures made of polysaccharides, proteins or short peptides can be easily modulated in terms of physicochemical features and can potentially act as reservoirs of soluble factors for their fine controlled release. In this context, hydrogels are injectable polymer-based biomaterials able to generate 3D networks with a huge potential in biomedicine. Interestingly, there is a broad catalogue of hydrogels, since they can be formed by different natural or synthetic polymers. Their generic architecture, swelling properties, pore size, interconnectivity, morphology and mechanical properties can be modulated through the fabrication of homopolymers, copolymers, by interpenetrating, double networking or by choosing covalent or physical interactions for the network construction [(106)]. Although hydrogels show by themselves interesting properties as scaffolds for tissue engineering, many efforts have been devoted to the development of artificial extracellular matrices based on hydrogels that offer not only mechanical but also biological cell-instructive cues, such as the targeted presentation of GF [(107,108)].

Hydrogel-based GF release. GFs have critical roles in cell proliferation, differentiation and survival, being the main source of biomolecular cues in any tissue engineering approach. Specifically, BMPs, transforming GF (TGFs), vascular endothelial GF (VEGF), fibroblast GF (FGFs), nerve GF (NGF) and insulin-like GF (IGFs) are the most important soluble effectors

involved in the tissue regeneration processes [109] (Table 2). The administration of such GFs has already been tested through the injection of their soluble versions. However, first clinical trials have shown low efficiency due to uncontrolled delivery. In this regard, hydrogels have appeared as a promising scaffold for GF immobilization and sustained release [107]. On the one hand, through the immobilization of GFs in hydrogels, the stability as well as the specific spatio-temporal delivery of such key signaling molecules can be defined and improved. Moreover, further developments of such delivery platforms are expected to reduce the doses of GF to reach the desired effect. On the other hand, the embedment of such GF in hydrogel matrices allows the generation of biomaterials able to mimic both the biological and physico-chemical functions of the extracellular milieu.

GFs (or any other drug of interest) can be immobilized in scaffolds and matrices by simple dispersion or physical entrapment, but also via biochemical or covalent links between the molecule of interest and the scaffold [110]. Since during the immobilization process, bioactivity of the protein or other drugs might be affected, a pre-treatment with poly-ethylenglycol (PEG) and an optimal buffer for drug encapsulation in micro- or –nanoparticles can notably improve the resulting bioavailability and bioactivity. Once immobilized, GFs are released from the scaffold by simple diffusion or *via* degradation of the polymeric matrix [109]. Promising prototypes of biomimetic hydrogels designed for GF delivery have been generated for skeletal regeneration, angiogenesis and vessel formation, and nerve regeneration, among others. Regarding bone tissue repair, different authors have proven the potential of different types of structures namely gelatine-poly(propylene)-based hydrogels [111], hybrid hydrogels [107], fibrin-based hydrogels [110] and alginate-based hydrogels [112,113,114], among others, combined with GFs. Interestingly, many of these studies have used GF-loaded microspheres to increase protein stability [111,107], observing promising results *in vitro*.

Hydrogels for bone and cartilage regeneration. The combined delivery of TGF-β3 and BMP-2 incorporated in alginate hydrogels is highly efficient and more effective for bone regeneration than free versions [112]. In agreement, the potential of GF release from hydrogels to regenerate bone tissue has been also demonstrated in rat, rabbit, sheep and dog [110]. As a complementary approach, the addition of integrin binding sites adjacent to GF-binding sites improves the final result [19]. Very recently, the generation of a novel and improved type of GF-releasing hydrogel has been described, able to stimulate *ex vivo* bone development and tissue repair [113,114]. Specifically, the authors have used a novel decellularized, demineralized bovine bone extracellular matrix combined with an alginate hydrogel as scaffold. Besides, this biomaterial was decorated with microparticles containing GFs and capable of releasing such GFs in a temporal and controlled manner. Individually, VEGF enhances cell migration, TGF-β3 stimulates cell proliferation, and BMP-2 specifically enhances collagen deposition. However, dual combinations of these GFs show a synergic effect, being possible to simultaneously induce migration and collagen deposition when using VEGF and BMP-2, and observing a greatest influence on tissue deposition when combining TGF-β3 and BMP-2 [113,114]. Furthermore, engineered hydrogels can be used to stimulate cartilage regeneration [115]. Considering that MSCs are a promising source for cartilage regeneration and that TGF-β family has a key role in the chondrogenesis process of MSCs, Jung and collaborators have designed Cyclodextrin(CD)-

and Cucurbituril(CB)-based hydrogels able to deliver TGF-β through a spatiotemporal control *in vivo*, showing again promising results [115].

Hydrogels for angiogenesis. Since angiogenesis is clearly dependent on the activity of GFs such as VEGF, FGFs and angiopoietin-1, hydrogels have a high potential as GF releasing systems also in this field. PEG-based hydrogels, combined with covalently-linked VEGF, have shown the ability not only to stimulate cell migration and proliferation, but also to maintain their viability [116,117]. Interestingly, it has been observed, as it has previously mentioned for skeletal tissue regeneration, an improved effect of RGD motifs when administered simultaneously to two GFs in both a chicken chorioallantoic membrane (CAM) assay and in an *in vivo* model [117]. In line with these studies, Thomas and co-workers have developed an advanced type of PEG hydrogels for localized and sustained delivery of angiogenic factors, using immobilized lentiviruses as GF expression system. Specifically, virus particles were incorporated in heparin-chitosan nanoparticles, which were finally immobilized onto a PEG porous structure. Interestingly, an increase of endothelial cells and blood vessels around the hydrogel used was observed using both *in vitro* and *in vivo* approaches [118].

Hydrogels for nerve tissue regeneration. Although the peripheral nervous system has a regenerative potential after nerve injury this not ensures complete tissue regeneration and in this context, hydrogels have also been used as controlled GF delivery platforms. For instance, a PLA-b-PEG-b-PLA microgel loaded with NGF and gelatin-based hydrogels loaded with VEGF [119] have been extremely efficient. Microgels, which can be directly injected into the tissue, have shown the ability to release NGF through 22 days *in vitro* [120]. On the other hand, Gnavi and collaborators have evaluated VEGF release from gelatin-based hydrogels using Schwann cells and Dorsal root ganglia (DRG) explants (models for glia and motor neurons), concluding that this biomaterial induces neurite outgrowth from DRG explants [119].

Amyloid engineering. Many protein and peptides have the natural capability to generate supramolecular fibrillar structures rich in beta-sheet conformation and stabilized by noncovalent interactions. This fact can be explained by the inherent physicochemical features of the protein and peptide backbone which exhibits a high propensity to establish hydrogen bonds, allowing the growth and stability of the protein/peptide amyloid fibers [121]. These amyloids, although firstly regarded as hazardous elements in several pathologies such as Alzheimer, Parkinson or Huntington disease are increasingly showing promise in the field of biomaterials as self-assembly protein fibrillar scaffolds. The capacity of generating a fibrillar matrix, resembling in composition and conformation the natural ECM, has focused the interest for this type of material in regenerative medicine, tissue engineering and other medical applications [122]. Additionally, the discovery of functional amyloids by Maji and co-workers, as reservoir of more than 30 releasable human hormones, opens the possibility of reaching a tight spatial and functional control of protein release from amyloid materials [123]. In this regard, amino acid stretches forming the amyloid building blocks can be chemically modified to finely regulate the disassembly of the fibrillar matrix. In particular, light-triggered release of the amyloid fibril building blocks can be an appealing and versatile approach. Measey et. al have provided evidence of how a simple substitution of lysines by a photocaged variant lys(Nvoc) allows the generation of amyloid fibrils with the capacity of disassembly upon irradiation with UV light. The lysNvoc light-mediated cleavage release the moiety attached to

the side chain, restoring the regular amino acid properties and providing a significant change in the hydrophobicity that causes the disassembly of the amyloid fibril [124].

Protein release from amyloid scaffolds. The plasticity shown by functional amyloids could be combined with other intrinsic features such as the slow release of the forming protein fibril. Elegant studies have tested the potential application of this platform for a sustained delivery of therapeutic proteins and peptides. This property would allow a significant improvement in treatments that require recurrent administration of the active molecule such as chronic or long-term diseases. In this context, amyloids formed by insulin or gonadotropin-releasing hormone (GnRH) analogues have been successfully designed. Thus, supramolecular insulin assemblies (SIA) have been proposed as a long-term solution to current administration of the soluble version. Luo and co-workers combined the SIA in Layer by Layer (LbL) films in order to obtain an insulin reservoir with very tight control of the molecule release. Subcutaneous implants of SIA structured in LbL films were applied to diabetic rats allowing the control of the glucose levels in an accurate manner during 295 days [125]. Similarly, analogues of the GnRH can be used for the control of numerous sex steroid dependent pathophysiologies. These peptides are able to self-assemble in amyloid supramolecular structures with distinct stabilities and releasing properties depending on the analogue forming the amyloid fibril. Besides, subcutaneous implantation of GnRH analogs allowed the increase in the duration of the treatment compared to their soluble counterpart [126].

Finally, bacterial IBs are functional amyloids that apart from the topographical potential for mechanical stimulation, cell adhesion and guidance discussed above, show high penetrability in mammalian cells and release the forming protein, in a functional form, inside cultured cells. These particles can be employed to add functionalities to 2D and 3D cell culture materials and exhibit a high versatility regarding the forming protein, its biological activity, and the physicochemical properties of the whole particle [127,128]. Such functionalized surfaces support the intracellular delivery of biologically active proteins to up to more than 80 % of the colonizing cells, in a process slightly influenced by the chemical nature of the scaffold. The interesting combination of 3D soft scaffolds such as PLA and protein-based sustained release systems such as bacterial IBs (Bioscaffolds) (Table 2), offers promise in the fabrication of fully bio-inspired hybrid matrices for multifactorial control of cell proliferation in tissue engineering under complex architectonic setting-ups [129,130,131,132]. Although the uses *in vivo* of IBs and other amyloid materials could be restricted by their potential toxicity and immunogenicity, the growing amounts of promising data obtained in cell culture and the emerging concepts around functional and non-toxic functional amyloids [133] prompt to envisage wide usability in *ex vivo* applications. Also, the absence of organic toxicity in oral administration of high IB doses [134], the possibility to obtain these materials in LPS-free cell factories [135] and the planned use of these particles to deliver homologous proteins such as GFs (then being formed by such homologous proteins) should ensure biocompatibility and minimize potential immune reactions in *in situ* tissue regeneration.

Drug release from functionalized amyloids. Apart from the direct release of the amyloid building blocks, these matrices can be also functionalized by soluble effectors, expanding the possibilities of action by providing multiple stimuli to the target cells (Figure 1). In this regard, simple co-incubation of the soluble effector during the supramolecular structure formation can

be enough in order to entrap the soluble factor that would be progressively released during the degradation of the matrix. Following this principle, Chun et al incorporated retinoic acid to a K-casein amyloid hydrogel in order to gain control over neuronal differentiation [136]. Other approaches consist in modifying the backbone of the amyloid building block to incorporate a tag with a high affinity for a complementary motif, fused to the soluble factor. Specifically, the biotin-streptavidin pair has been successfully explored in order to functionalize amyloid fibrils without altering their fibrillar structure. Incorporating biotin to the peptide forming the amyloid fibril as well as to the IGF-1 and linking both through a multivalent streptavidin, Davis and co-workers obtained amyloids capable to improve cardiac myocyte survival and growth. Thus, culturing *ex vivo* these cardiac myocytes on IGF-1 functionalized amyloids improved their further response upon implantation of the biomaterial in rat models of myocardial infarction [137].

Peptide amphiphiles. Additionally to protein and peptide organization in amyloid structures, other molecules with peptidic nature confer extra levels of complexity to fibrillar architectures. In this regard, peptide amphiphiles (PA), generally composed by a peptidic hydrophilic head and an alkyl hydrophobic tail, allow the formation of micelles, fibers or even hollow tubes depending on the concrete nature of the PA used. Thus, we can find PA including cyclic peptides [138], bolaamphiphiles [139], surfactant-like peptides [140] or aromatic dipeptides [141]. In general terms, the hydrophilic head is exposed to the solvent while the hydrophobic tails are disposed avoiding the contact with the solvent. As it happens with amyloid fibrils, PA self-assembly and stability are also due to non-covalent interactions. PA fibers allow a higher plasticity in the fiber supramolecular organization although their building blocks cannot act as the own soluble effectors and should be functionalized. PA self-assembled fibers have also proved their applicability as biomaterials. In this sense, PA fibers functionalized with IKVAV or RGD peptides are able to influence neuronal cell alignment and migration. Neuronal cell alignment onto these matrices rendered enhanced neurite growth in both *in vitro* and *in vivo* assays [142]. PA fibers also functionalized with the neuroactive peptide IKVAV were able to partially restore cognitive impairment upon PA-IKVAV injection into the hippocampus in Alzheimer's disease mice model. This result was linked to markedly neurogenesis observed in treated mice. Additionally, Yang and co-workers observed a decrease in the levels of soluble aβ-40 aβ-42 and amyloid-β plaques probably derived from the IKVAV functionality [143].

Table 2. Representative protein materials used for an integrated biological control of cell proliferation.

Material	**Presentation**	**Application**	**Reference**
Mussel adhesive proteins	Scaffold coated	Enhances cell adhesion, proliferation and osteogenic formation in vitro and in vivo	[102]
Silk fibroin	Electrospun matrices	Enhances adhesion of different cell types in vitro	[101]
Fibronectin	Coating surfaces	Enhances adhesion and retains regenerative capacity of human hematopoietic Stem Cells ex vivo	[144]
Laminin E8	Coating surfaces	Support efficient	[145]

fragment		adhesion and expansion of dissociated human pluripotent stem cells	
Vitronectin	Coating surfaces	Promote adhesion and osteogenic diferenciation of human mesenchymal stem cells	[146]
GFOGER	Coating synthetic poly-caprolactone (PCL) scaffolfs	Increase and accelerate bone formation in critically-sized segmental defects in rats	[147]
Hialuronic acid (HA)	HA-binding scaffold	Improved cartilage tissue production in a rat knee osteochondral defect model	[148]
Elastin-like polypeptides (ELP) with fibronectin cs5 and a proteolytic domain	Absorbed to glass surfaces	Enhance epithelial cell attachment, proliferation, and retention of the differentiated phenotype in ocular surface tissue engineering	[149]
Bone sialoprotein	Surfaces coated with the oligomerized form	Promote osteoblast adhesiveness	[150]
Osteopontin	Hydroxyapatite nanoparticles functionalized with osteopontin (OPN) in a matrix of poly-D,L-lactic-acid (PDLLA)	Increased the formation of new bone in the porosities of a canine implant	[151]
TGF-β	Cyclodextrin(CD)- and Cucurbituril(CB)-based hydrogels	Effective chondrogenic differentiation	[115]
VEGF	PEG-based hydrogels	Stimulates cell migration and proliferation and maintain cell viability	[116,117]
VEGF	Embedded in gelatine-like gels	Nerve grow stimulation in in vitro models	[152]
NGF	PLA-b-PEG-b-PLA microgel	Material with potential to maintain NGF activity in the peripheral nervous system	[153]
BFGF-2	In surface-coated amyloidal micro-particles	Stimulation of cell proliferation in 2D and 3D scaffolds	[132]
TGF-β3 and BMP-2	Embedded in alginate hydogels	Bone regeneration in in vivo models	[112]
TGF-β3 and BMP-2	Embedded in alginate hydogels	Improves tissue deposition	[113]
VEGF and BMP-2	Embedded in alginate hydogels	Enhances migration and collagen deposition	[113,114]
Supramolecular insulin assemblies (SIA)	LbL films	Long term control of glucose levels	[125]
GnRH analogues	Amyloid fibers	Treatment of sex-steroid dependent pathophysiologies	[126]
Retinoic Acid – K-Casein	RA functionalizing amyloid K casein hydrogel	Neuronal differentiation	[136]
IGF-1	Functionalizing amyloid fibrils	Improve cardiac myocyte survival and growth in	[137]

		infarctation rat models	
Bacterial inclusion bodies	Functionalizing 2D and 3D cell culture scaffolds	Show promise as protein drug delivery platform	(127)
IKVAV or RGD	Functionalizing PA fibers	*In vitro* and *in vivo* neurite growth stimulation	(142)
IKVAV	Functionalizing PA fibers	Restores cognitive impairment in Alzheimer's disease	(143)
IKVAV	Immobilized on different dextran-coated materials	Promote substantial neuron cell adhesion and neurite outgrowth, and minimal fibroblast and glial cell adhesion	(154)

Future perspective

The comprehension of how cells respond to their environment, regarding the set of complex mechanical, biomechanical and biological stimuli provide by the ECM, is gained by the analyses of individual effectors or effector categories and their impact on cell biology. Then, 2D and 3D scaffolds are under development to offer appropriate surface roughness at both nano and micro scales to stimulate cell attachment, proliferation and differentiation and to modulate cell positioning and fate in regenerative medicine. In parallel, protein materials with appropriate biological and mechanical properties (such as adhesiveness) might serve for coating complicated scaffolds to provide more cell-friendly supports. In an attempt to integrate the range of stimuli, soluble protein factors, usually available upon external supply, are being incorporated into new-generation scaffolds, which act as topographies and simultaneously as drug reservoirs for sustained release along the tissue formation process. The expected gain of a temporal control over the activity of such hybrid materials and the resulting factor supply will necessarily conduce to designing smarter, bio-inspired scaffolds for a more accurate and integrative mimicry of cellular environment and a tighter control of tissue formation in artificial but biomimetic settings.

Executive summary

Engineering scaffold topography Engineering surface topology by either top-down or bottom-up approaches offers a convenient way to modulate cell functions critical for tissue engineering such as attachment, proliferation, positioning, migration and differentiation.

Lithographic techniques are powerful instruments to topographically control cell behavior in modified scaffolds.

As a surface engineering method, particle deposition remains, instead, less dependent on the surface material chemistry and offers a high versatility in the control of patterning.

Diverse materials and biomaterials are suitable for the fabrication of biocompatible 2D and 3D scaffolds.

Surface-engineered 3D scaffolds offer extremely interesting settings for the structural mimicry of the ECM.

Tailoring bio-functional protein materials

A set of natural or modified proteins and peptides allow coating of unfriendly materials for efficient cell colonization, enabling the applicability in tissue engineering of xenobiotic materials that are appealing from the fabrication point of view.

Developing functional drug-releasing biomaterials

Hydrogels and other polymeric materials adapted as 3D scaffolds for tissue engineering can be loaded with soluble protein factors with impact in tissue formation, for sustained release during cell colonization.

Hydrogels can be tuned and functionalized for very precise specific applications in tissue engineering such as nerve tissue regeneration and angiogenesis.

Full-length proteins resulting from biofabrication and natural or synthetic peptides are being engineered to form bio-inspired matrices for fast and efficient cell colonization.

Amyloidal materials are specifically suitable for the slow release of their bio-active building blocks, mimicking the performance of hormone secretory granules in the endocrine system and avoiding the external supply of soluble factors.

The combination of both topography and biological effectors in these protein materials offer a refreshing approach to the generation of bio-inspired, bio-active substrates (bioscaffolds) for tightly controlled, self-contained tissue engineering platforms.

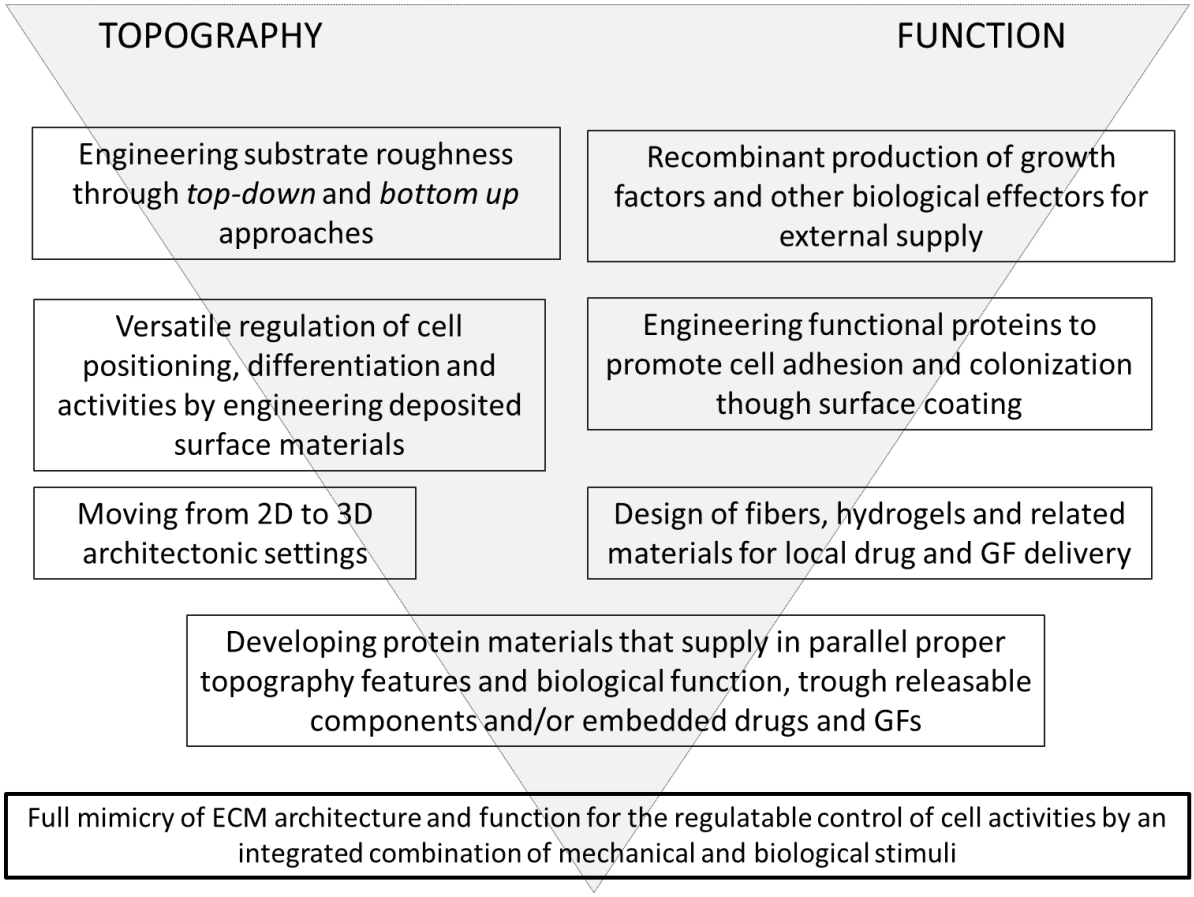

Figure 1. Tissue engineering requires both addressing substrate topography requirements at the cell sensing scale and providing appropriate biological stimuli for proper cell adhesion, positioning, migration, proliferation, differentiation and activity (top). This is intended to finely replicate *ex vivo* and *in vivo* features of the ECM in synthetic platforms, to finely regulate cell behavior in a targeted way (bottom). Plain engineering of surface topography through *top-down* and *bottom up* approaches have generated numerous insights about how cells respond to mechanical stimuli. On the other hand, soluble factors (mainly proteins) are progressively incorporated to 3D scaffolds for their local release, while proteins themselves are exploited for their adhesive properties and architectonic potential in form of coating layers, fibers, hydrogels and amyloids. Although still far from clinical implementation, bioinspired, hybrid and homogeneous protein materials are showing themselves as promising alternatives to separated topographical and biological effectors for an integrative manipulation of cells in tissue engineering.

Acknowledgments

The authors acknowledge the financial support received for the design of protein-based materials from FIS (to E.V., PI12/00327), from La Marató de TV3 (to A.V., TV32013-132031 and to E.V. TV32013-133930), from INIA (to E.G.F., RTA2012-00028-C02-02), from MINECO (to A.V., BIO2013-41019-P) and from the Centro de Investigación Biomédica en Red (CIBER) de Bioingeniería, Biomateriales y Nanomedicina (NANOPROTHER project), financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. We are also indebted to the Protein Production Platform (CIBER-BBN-UAB) for helpful technical assistance (http://www.ciber-bbn.es/en/programas/89-plataforma-de-produccion-de-proteinas-ppp). A.V. has been distinguished with an ICREA ACADEMIA Award.

Selected articles:

Of special interest:

Clark, P., Connolly, P., Curtis, A. S., Dow, J. A., & Wilkinson, C. D. (1990). Topographical control of cell behaviour: II. Multiple grooved substrata. Development, 108(4), 635-644.
One of the first uses of photolithography for preparation of grooved substrates with controlled geometry on the cell guidance.

Chen et al. Towards delivery of multiple growth factors in tissue engineering. Biomaterials. 2010. 31: 6279-6308.
In this article, the authors introduce the concept of dual and multiple delivery of growth factors for tissue engineering using several examples already published.

Dalby, M. J., Riehle, M. O., Johnstone, H., Affrossman, S., & Curtis, A. S. G. (2002). In vitro reaction of endothelial cells to polymer demixed nanotopography. Biomaterials, 23(14), 2945-2954.
Study of endothelial cell response to nanotopography obtained by poymer demixing. It is demostrated, that morphological and cytoskeletal response depends on topographical feature size.

Reichl S 2009. Films based on human hair keratin as substrates for cell culture and tissue engineering. Biomaterials 30(36): 6854–6866.
This is a seminal article on the use of human hair keratin as a coating material for cell culture using novel methods for film generation and performing an exhaustive study on cell growth and epithelial tightness of different cell types.

Stukel et al. Polyethlyene glycol microgels to deliver bioactive nerve growth factor.J Biomed Mater Res A. 2014 Apr 26. doi: 10.1002/jbm.a.35209. [Epub ahead of print]
This paper is an interesting in vivo example of the use of hydrogels as delivery platforms of growth factors with promising results in nerve tissue regeneration.

Yang Z et al. 2012. Mussel-Inspired Coating of Polydopamine Directs Endothelial and Smooth Muscle Cell Fate for Re-endothelialization of Vascular Devices. Advanced Healthcare Materials 1(5): 548–559.

This is a nice example on the use of polydopamine (PDAM), a mussel adhesive protein inspired coating, as a vascular stent surface to promote endothelial function with a deep biocompatibility analysis.

Yang,H., Qu,T., Yang,H., Wei,L., Xie,Z., Wang,P., Bi,J., 2013. Self-assembling nanofibers improve cognitive impairment in a transgenic mice model of Alzheimer's disease. Neurosci. Lett., 556, 63-68.
Interesting in vivo example of PA nanofibers functionalization with the IKVAV motif.

Of outstanding interest:

Dalby, M. J., Gadegaard, N., Tare, R., Andar, A., Riehle, M. O., Herzyk, P., Wilkinson C. D. W.& Oreffo, R. O. (2007). The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. Nature materials, 6(12), 997-1003.
Demonstration that the proper use of nanoscale modification of scaffolds stimulates human mesenchymal stem cells (MSCs) to produce bone mineral in vitro, in the absence of osteogenic supplements.

Maji,S.K., Perrin,M.H., Sawaya,M.R., Jessberger,S., Vadodaria,K., Rissman,R.A., Singru,P.S., Nilsson,K.P., Simon,R., Schubert,D., Eisenberg,D., Rivier,J., Sawchenko,P., Vale,W., Riek,R., 2009. Functional amyloids as natural storage of peptide hormones in pituitary secretory granules. Science, 325, 328-332.
In this work the authors unveil for the first time a number of peptide hormones being stored in releasable amyloids in the pituitary secretory granules. These functional amyloids evidence the need of rethinking the relationship between amyloids and amyloidal toxicity.

Smith et al. Evaluation of skeletal tissue repair, Part 1: Assessment of novel growth-factor-releasing hydrogels in an ex vivo chick femur defect model. Acta Biomater. 2014 Oct;10(10):4186-96. doi: 10.1016/j.actbio.2014.06.011. Epub 2014 Jun 14.

Smith et al. Evaluation of skeletal tissue repair, Part 2: Enhancement of skeletal tissue repair through dual-growth-factor-releasing hydrogels within an ex vivo chick femur defect model. Acta Biomater. 2014 Oct;10(10):4197-205. doi: 10.1016/j.actbio.2014.05.025. Epub 2014 Jun 4.
Both articles describe the use of a new and improved type of hydrogel for tissue engineering applications. This hydrogel has been combined, for the first time, with a decellularized and demineralized extracellular matrix and microparticles containing GFs with the aim to successfully mimic both the biological and physico-chemical functions of the extracellular environment.

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