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Review Article

# Biomimetic Nanostructured Materials — Potential Regulators for Osteogenesis?

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## Abstract

**Nanostructured materials are gaining new impetus owing to the advancements in material fabrication techniques and their unique properties (their nanosize, high surface area-to-volume ratio, and high porosity). Such nanostructured materials mimic the subtleties of extracellular matrix (ECM) proteins, creating artificial microenvironments which resemble the native niches in the body. On the other hand, the isolation of mesenchymal stem cells (MSCs) from various tissue sources has resulted in the interest to study the multiple differentiation lineages for various therapeutic treatments. In this review, our focus is tailored towards the potential of biomimetic nanostructured materials as osteoinductive scaffolds for bone regeneration to differentiate MSCs towards osteoblastic cell types without the presence of soluble factors. In addition to mimicking the nanostructure of native bone, the supplement of collagen and hydroxyapatite which mimic the main components of the ECM also brings significant advantages to these materials.**

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**Key words: Biomaterials, Biomimetic, Bone, Hydroxyapatites, Nanomaterials, Stem cells, Tissue engineering**

## Introduction

Bone is the second most common transplantation tissue after blood. Globally, at least 2.2 million of bone grafting procedures are performed annually and approximately 500,000 of such procedures are done in the United States (US) alone.[1-3] Figure 1 shows the orthopaedic industry by market segmentation in the US.[4] It is estimated that the orthopaedic market is set to generate revenues of over US$20 billion in 2010. The US being the biggest player is said to contribute 59% of the total world orthopaedic market shares.[4] Bone graft market alone is valued over US$2.5 billion.[5]

The ideal bone graft should possess the 3 properties namely osteoconduction, osteogenesis and osteoinduction. Osteoconduction is the ability of biocompatible scaffolds to promote the attachment, survival, migration, and distribution of ostegogenic cells. Osteogenic graft materials contain osteogenic stem cells or progenitors to create new bone through the differentiation process. Lastly, osteoinductive bone grafts contain soluble or matrix-bound signals to initiate stem cells or progenitors towards osteoblastic cell type.[6,7]

Currently, autogenous and allogeneic bone grafts are the most common approaches for bone defects treatment. However, these sources of bone grafts have significant disadvantages including limited supplies, the hazard of adverse immunological response and pathogenic transmission.[8,9] So, synthetic bone grafts (usually calcium phosphate-based) provide an alternative bone graft option. Growth factors (e.g. bone morphogenetic protein-2 or -7 (BMP-2, BMP-7)) can be incorporated to improve their osteoinductive capabilities. The main drawbacks of these synthetic materials are that they are brittle, possess low

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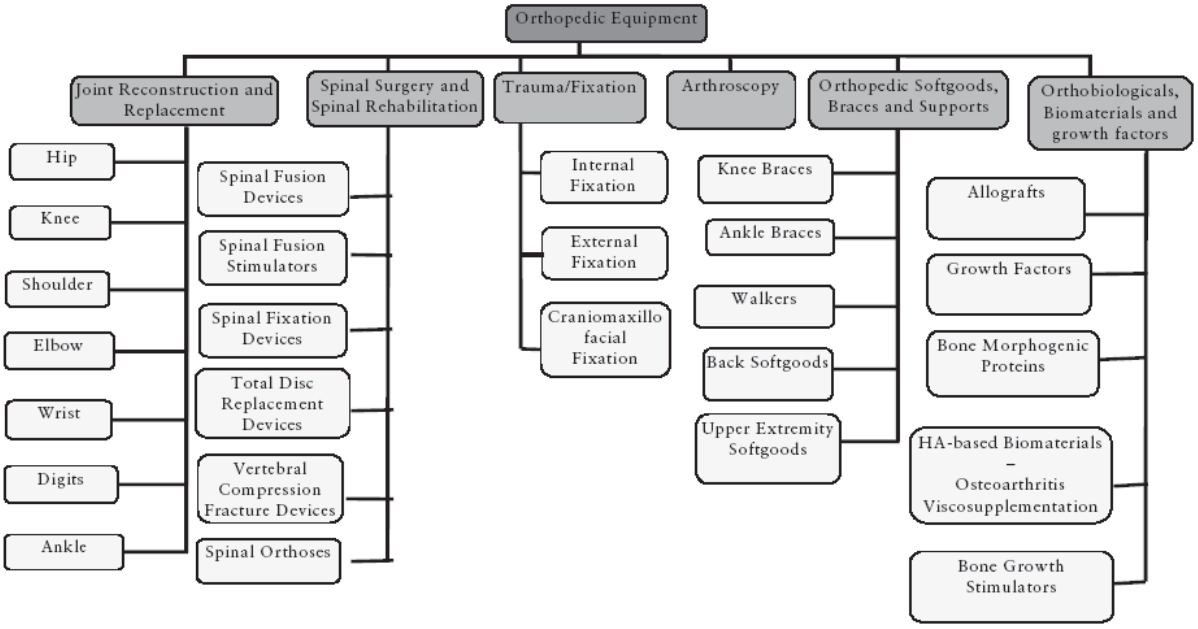



Fig. 1. Orthopaedic industry by market segmentation in the US.[4]

mechanical strength; and depending on their fabrication methods, they can be highly crystalline (due to sintering at very high temperatures of more than 1000ºC). Additionally, most biomaterials have poor surface interaction with the host tissue, resulting in the lack of adequate tissue formation around the biomaterials.[10] Besides, some materials act only as passive scaffolding, so insufficient remodeling occurs.[10] These phenomena may be caused by the fact that structural and composition properties of those materials do not resemble those of natural bone. Current bone graft systems are usually blended systems and mimic native bone only at a micro-level, such as HEALOS® Bone Graft Replacement, CopiOs® Bone Void Filler, Osteopore® PCL scaffold Bone Filler, etc. To solve those issues, many recent studies have focused on nanostructured materials which mimic the native bone at nano-level.

One of current challenges in bone tissue engineering is how to develop osteoinductive graft materials to differentiate stem cells towards osteoblasts without the presence of soluble factors. Biomimetic structured materials have been expected to do that. In this review, we summarise recent studies which have provided evidence of these materials as potential regulators for osteogenesis.

## Mesenchymal Stem Cells for Bone Regeneration

Work in the last decade includes evidence that stem cells possess self-renewal, multi-lineage differentiation and *in vivo* functional capabilities. Stem cells of interest include mainly embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs). Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of blastocyst-stage 5-day embryo.[11] They possess high proliferative capability,[12,13] are able to form 3 embryonic germ layers (endoderm, mesoderm and ectoderm),[11] produce germline chimaeras,[14] exhibit differentiation in teratomas[11] and express specific ESC markers.[11] However, the safety and efficacy of hESC lines may be a concern. These include technical issues such as potential of hESC rejection and the risk of tumorigenicity. There are also ethical and religious issues involving the harvesting of donor oocytes and destruction of the blastocyst.

As such, MSCs provide an attractive alternative to ESCs and these cells can be readily obtained with less controversy from bone marrow,[15] umbilical cord blood[16] and adipose tissue.[17] A recent study shows that the bone nodules that are formed by osteoblasts and MSCs exhibit the hallmarks of native bone, whereas those are formed by ESCs differ in terms of composition, stiffness and nano-architecture.[18] More importantly, MSC has a versatile differentiation profile. Autologous MSCs surmount immune rejection and carcinogenesis is minimised.[19] Several reports stated that MSCs facilitate bone repair.[20-22]

MSCs are able to differentiate into many cell types such as adipocytes, chondrocytes, osteoblasts and myocytes.[15] Under suitable stimuli, MSCs can be initiated to differentiate into osteoblastic cell types. This process is known as

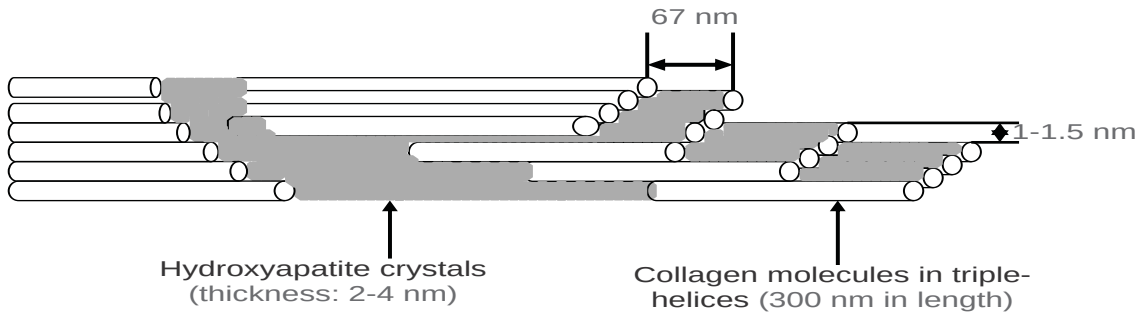



Fig. 2. Schematic of mineralised collagen fibrils of bone.

osteogenic differentiation. The use of growth factors such as BMP and fibroblast growth factor (FGF)[22-24] and osteogenic supplements (dexamethasone, β-glycerophosphate, ascorbic acid, vitamin D)[25,26] are some approaches which aim to induce osteogenic differentiation. In addition, others have illustrated the benefits of culturing more than one cell type (co-culture) to aid in osteogenic differentiation.[27] In this review, not such growth factors/ osteogenic supplements, but biomimetic nanostructured materials will be emphasised to indicate their role in the osteogenic differentiation of MSCs.

## Strategy for the Design of Bone Graft Materials

The key tenet of tissue engineering is to regenerate diseased, damaged tissue or organ using biodegradable materials including synthetic or natural polymers. Examples of synthetic polymers for potential bone applications include polycaprolactone (PCL),[28] poly(L-lactide) (PLLA),[29] poly(D, L-lactic-co-glycolide) (PLGA)[30] and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV).[31] Others have used natural polymers such as collagen,[32] chitosan,[33] alginate,[34] agarose[34] and silk[35] in the quest for developing better bone graft materials.

The understanding of material science together with stem cell biology and signaling pathways (e.g. mitogen-activated protein kinase (MAPK) and phosphatidyl inositol-3-kinase (PI3K) etc.) is important to expedite expansion and differentiation of stem cells into tissue-specific lineages without changing the plasticity nature of the stem cells. Various biomaterial fabrication techniques aim to construct a microenvironment or niche similar to that in the body. During trauma and disease conditions, loss of tissue may occur and instead of being in homeostasis state, the stem cells migrate out and start their proliferative and differentiation work at the damaged site. At this site, stem cells stored in the niche are exposed to an array of soluble chemokines, cytokines, growth factors, as well as insoluble transmembrane receptor ligands and ECM proteins.[36] ECM not only provides the structural and functional aspects of bone, it also provides key regulatory signals for cell proliferation and differentiation by cell-receptor interactions, mediating the diffusion of soluble growth factors and transmitting and attenuating mechanical signals.[37]

Understanding the composition, architectural, biophysical and mechanical properties of native bone would give us great insights in designing bone grafts for various applications. Bone ECM is a nanocomposite with an intricate hierarchical structure, assembled through the orderly deposition of nano-hydroxyapatite (HA) within a type I collagenous fibril matrix. Collagen molecules are triple helices with a length of about 300 nm. The HA mineral crystals are embedded parallel to each other and parallel to the collagen fibrils, in a regularly repeating, staggered conformation (Fig. 2). Besides, bone is a nanocomposite where cells reside on ridges, grooves, pores and fibers of the extracellular matrix (ECM). The explosion in research towards designing nanocomposites for bone grafts are directed at polymeric nano-scale materials which closely mimicking the native bone structure. One can envisage that cellular interactions and behaviour such as adhesion, proliferation and differentiation on these nanotextured materials will be tremendously improve the osteogenic potential of these nanocomposites.

## Biomimetic Nanostructured Materials

Since the conceptual approach is to mimic native ECM, nanofibrous scaffolds (NFS) have been widely used recently to mimic the protein nanofibrils in the native ECM. Currently, there are 3 common methods for the fabrication of nanofibrous structures: self-assembly, phase separation and electrospinning. Among these techniques, self-assembly is the most complex technique, and able to construct nanofibres with very small diameters (a few to 100 nm). Phase separation is much simpler than self-assembly, and able to process many biodegradable and biocompatible polymers with diameters of 50 to 500 nm. However, the common constraints of these 2 techniques are that only short strands of nanofibres are produced, and it is really difficult to obtain nanofibres throughout a large

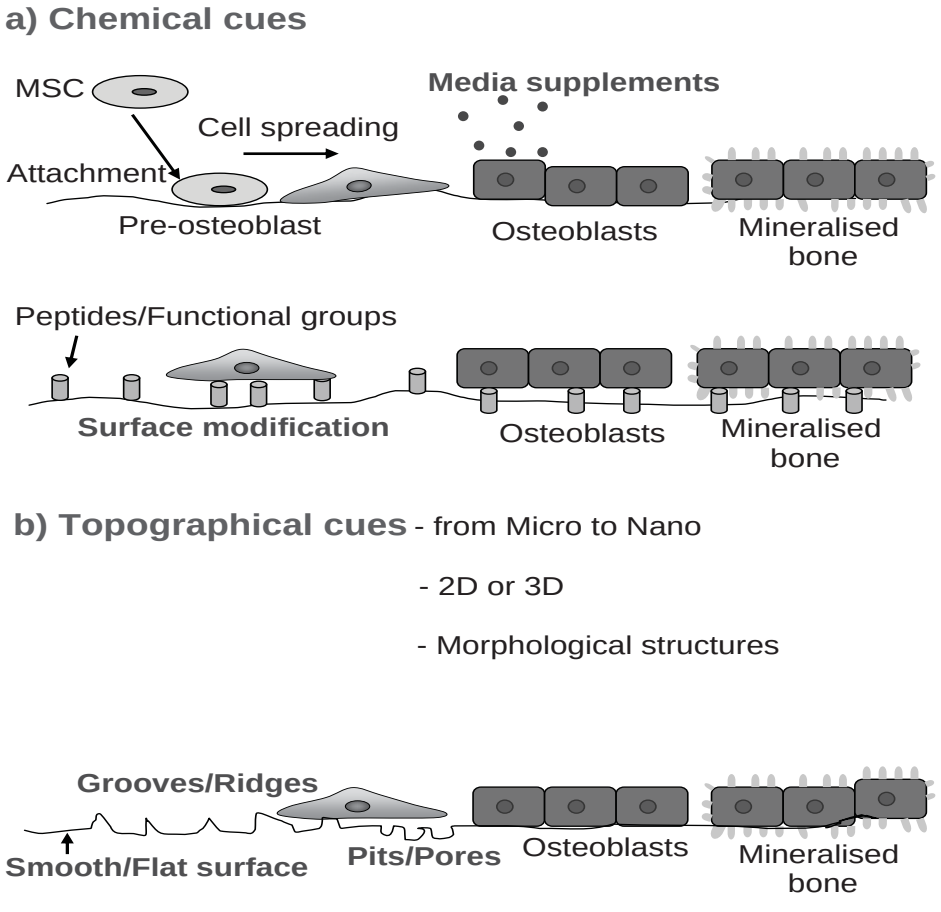
scaffold. Electrospinning is a technique used to fabricate polymeric nanofibers by means of an electrostatic force. Electrospinning is a reliable method to fabricate long continuous strands of nanofibres with diameters in the range of 50 ÷ 1000 nm, and these fibrous diameters can be controlled with a rather small deviation. Its flexibility in terms of material selection and the ability to create various nanofibrous architectures (nonwoven fibre mesh, aligned fibre mesh, patterned fibre mesh and random 3-dimensional structures) have also made this process highly attractive for scaffold fabrication.[38-40] Recently, a technique for fabrication and remodeling of 3D hierarchically organised nanofibrous assemblies using a dynamic liquid support system has been developed.[41,42]

To mimic the nanocomposite nature of bone, newer compositions of synthetic bone graft substitutes attempt to resemble the nano-HA and collagen fibrils composition of natural bone. Collagen, as one of the ECM proteins plays critical role in bone mineralisation, thus collagen is a prime candidate material for tissue-engineered graft material. Type I collagen has been used in several commercial products such as Collapat II (Biomet Inc.), Collagraft (Zimmer Inc.), Healos (Depuy Spine Inc.). Note that the above-mentioned commercial products are not tissue-engineering nanofibrous scaffolds. As collagen has a rapid adsorption rate and possess weak mechanical strength, polymer additions are often incorporated for enhancing the mechanical properties of the material constructs. Besides, polymers by themselves lack cell recognition signals,[43] and the addition of collagen provides the necessary binding sites for cell-material interactions. Polymer and collagen can be co-blended and then fabricated into nanofibrous scaffolds via electrospinning.[40] In electrospinning a high voltage field is applied to electrically-charge a liquid (material of interest: polymer, collagen, salts that can be fully dissolved in the appropriate solvents), resulting in nanofibres. Calcium salts such HA,[29] β-tricalcium phosphate (β-TCP) and calcium carbonate ($CaCO_3$)[28] can also be incorporated to mimic the inorganic component of native bone and to improve the osteoconductivity of the material construct.

The reasons why these biomimetic nanostructured materials are considered as potential regulators for osteogenesis and their examples will be discussed in the following section.

**Potential Regulators for Osteogenesis**

Figure 3 shows that regulating stem cell fate can be



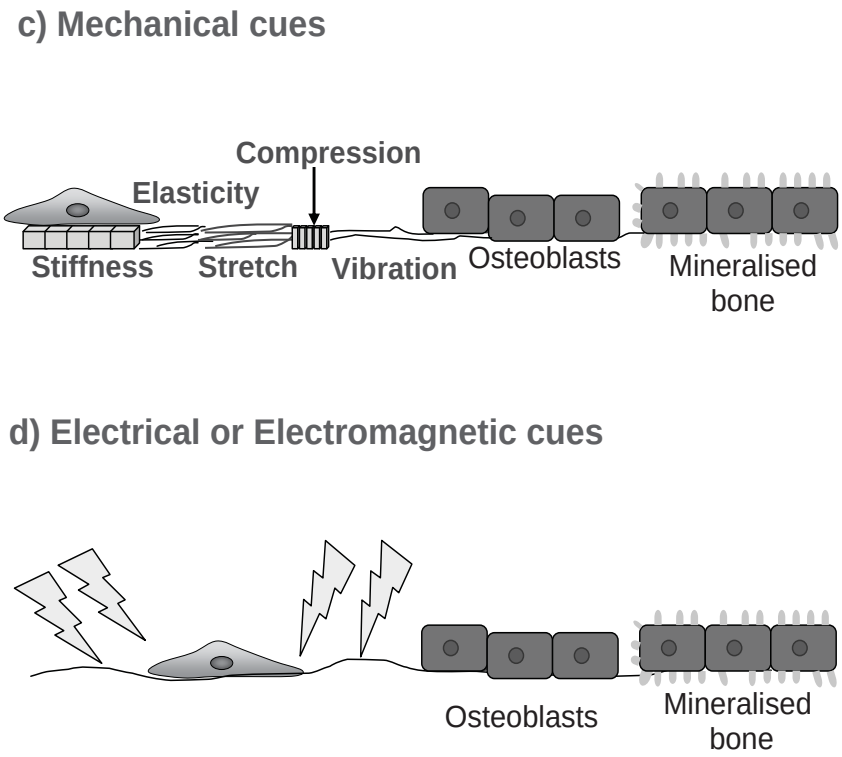



Fig. 3. Regulating cell fate through various cues. (a) Chemical (through use of media chemicals or surface modification), (b) Topographical (through surface features [such as pits, grooves, ridges, pores etc.], architectural form [2D vs 3D] or size effect [micro, nano scale]), (c) Mechanical (through various stress stimuli applied to substrate and/or cell construct) and (d) Electrical and electromagnetic cues (through application of electrical or electromagnetic currents/fields to stimulate substrate and/or cell construct).

achieved through various means, as such chemical, topographical, mechanical and electrical or electromagnetic cues. For chemical cues, media supplements or peptides/functional groups can be added into the environment to differentiate MSCs into osteoblasts. Besides, topographical cues such as size affect (micro/nano), architecture form (2D/3D) and morphological structures (pits/grooves/ridges/pores), etc are able to help MSCs differentiated. Additionally, various stress stimuli applied to substrate and/or cell construct, called mechanical cues, have been supposed to induce osteogenesis. Lastly, the differentiation of MSCs into osteoblasts can be stimulated by electrical and electromagnetic cues (through application of electrical or electromagnetic currents/fields to stimulate substrate and/or cell construct). In this review, we focus on the importance of topographical features and substrate characteristics of biomimetic nanostructured materials to inducing/enhancing MSC differentiation.

Nanofibrous scaffolds being in nanometer scale (in diameter) are said to resemble the ECM proteins, and such microenvironment is conducive for cellular interaction. Nanotexture is said to influence cell activity. Cells are subjected to topographical features such as protein folding, collagen bending within a niche *in vivo*. Nanoscale disorder has shown to stimulate osteogenic stem cell differentiation without chemical treatments.[44] Such geometric cues have demonstrated a dominant effect on adhesion, spreading, growth and differentiation of MSCs in several studies. Lateral spacing geometry of $TiO_2$ nanotubes of 30 to 50 nm was reported to be the critical threshold for cell fate.[45] Diameter (<15 nm) and spacing (<30 nm) was considered to be the effective length scale for augmenting integrin clustering and focal contact formation. Good evidence showed that smaller diameter nanotubes (15 nm) were associated with greater focal contact formation, stress fiber (contractile actomyosin bundles or actin filaments) assembly, cell spreading and osteocalcin differentiation compared to larger diameter nanotubes (100 nm). On the other hand, larger diameter nanotubes (>50 nm) resulted in the reduction in cellular activity, fewer focal contact and stress fibers and even programmed cell death.[45] In a separate study, it was shown that larger diameter nanotubes enhanced cell spreading compared to smaller diameter and

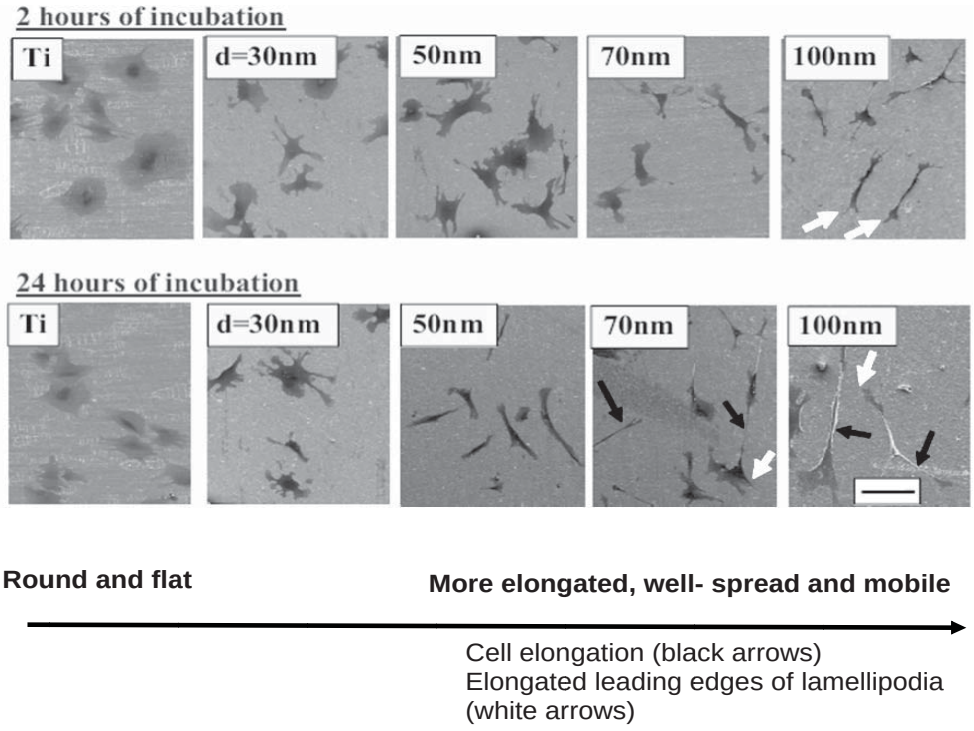



Fig. 4. SEM images of human MSCs on flat Ti and TiO2 nanotubes with diameters of 30 nm, 50 nm, 70 nm and 100 nm after 2hrs and 24 hrs of incubation.[46]

flat substrates as depicted in Figure 4.[46] Increased MSC adhesion on smaller diameter nanotubes was said to be due to the increased protein aggregates such as fibronectin and albumin. Conversely, larger diameter nanotubes increased osteogenic differentiation as cells were forced to elongate and stretch in search of protein aggregates, and such guidance and stressed-induced elongation resulted in osteogenic differentiation. Lower cell numbers were seen on larger diameter nanotubes within 24 hours, but after 7 days, the cell numbers for the different sized nanotubes were comparable, suggesting that the initial cell density could play a role in regulating the stem cell fate.[46] Increasing cell growth, cell numbers and osteogenic differentiation was also more evident 3D scaffolds with nanotextured surfaces compared to smooth 3D scaffolds,[47] implying the importance of nanotopographical features in modulating cellular activities. Besides, there is a central concept that cells attach and organise well on fibers that have diameters smaller than that of the diameter of the cells.[48]

Additionally, the high surface area-to-volume ratio and its high porosity (with small pore sizes) allow efficient nutrient delivery, gas exchange and waste excretion. One of the characteristics of nanoscale scaffolds is the enhanced absorption of biomolecules such as vitronectin on the scaffolds due to a high surface area-to-volume ratio,[49] which is important for example wound healing, creating a more favourable environment for cellular interaction. In addition, biomineralisation was significantly increased on nanofibrous scaffolds compared to solid-walled scaffolds.[50] For instance, when osteoblasts (bone cells) were seeded on both types of scaffolds, early bone markers such as runt-related transcription factor 2 (RUNX-2) protein and alkaline phosphatase (ALP) and middle-stage bone marker bone sialoprotein were higher on the nanofibrous scaffolds than on solid-walled scaffolds. Furthermore, the nanofibrous substrates seemed to promote protein adsorption such as fibronectin and vitronectin. Integrins associated with fibronectin ($\alpha v\beta 3$), vitronectin ($\alpha v\beta 3$) and collagen-binding ($\alpha 2\beta 1$) were enhanced on nanofibrous scaffolds compared to solid-walled scaffolds. This implies that substrates have an influence on osteoblastic phenotype and cellular signaling, suggesting the superiority of nanofibrous materials over solid-walled materials.[50]

Type I collagen which is used for co-blended nanofibres, proved to be a substrate for binding of BMPs[23] and is also chemotactic to fibroblasts, having high affinity cell-binding domains.[51] The activation of type I collagen specific integrins is said to have an osteogenic response to a bone cell line[52] and human bone marrow stem cells (BM-MSCs).[53]

Mineralised nanofibres, which mimic collagen fibrils and

nano-HA in native bone, elevated osteoblastic activities compared to non-mineralised nanofibres.[30] Figure 5 shows the nanotextured surfaces of mineralized nanofibres, where prominent groves and ridges are more evident on HA fibers than non-HA fibers.[30] The importance of closely mimicking the natural composition of bone can be delineated in several studies.[29,30,54] For instance, enhanced mineral deposition (57% higher) was observed when osteoblasts were grown on PLLA/Collagen/HA nanofibres compared to PLLA/HA nanofibres, suggestive of the synergistic effect of collagen and HA in osteogenic differentiation and bone mineralisation.[54] Many studies have shown that BM-MSCs are capable of differentiating towards an osteoblastic lineage.[32,37,55-57] It was also shown that when MSCs were cultured on HA surfaces, osteo-specific genes were up-regulated.[55,57] Not only the viability of human MSCs was not affected, the expression of ALP, osteogenic genes and calcium mineralisation of the MSCs were elevated when the cells were cultured on blended PLGA and nano-HA nanofibres.[58] It was speculated that when the cells interacted with HA, potent inductive substances were released. Using this conditioned media after the initial culture, uncommitted MSCs were then cultured without the presence of HA and upregulation of osteo-specific genes were observed.[57] Although some reports stated that HA induced osteogenic differentiation of MSCs and other cell types, there were also conflicting reports which saw an attenuation in osteogenic differentiation when cells were cultured on HA surfaces.[59,60] This could be due to the physical and chemical characteristics of the HA material such as crystallinity and particle size etc.[59]

A landmark paper highlighted the importance of matrix stiffness and its influence in directing MSC commitment towards a specific lineage.[61] Briefly, soft matrices were associated with neurogenic differentiation, stiffer matrices were corresponded to myogenic differentiation and lastly rigid matrices were related with osteogenic differentiation.[61] In a separate study, the stiffness of substrates (PEG-based materials) affected differentiation of pre-osteoblastic cells via mitogen-activated protein kinase (MAPK) activation.[62] It was reported that such ECM rigidity regulated osteogenic differentiation involving MAPK activation downstream of the RhoA-ROCK signaling cascade.[63] Early osteogenic differentiation markers, such as RUNX-2 and ALP expression were associated with stiffer materials.[62,63] The elasticity of the substrates also impinges upon cell proliferation, where stiffer substrates resulted up to 10-fold increase in cell numbers compared to lower stiffness substrates.[64] Interestingly, osteogenic differentiation of MSC were significantly increased on collagen-I coated substrates with the highest modulus,[64] suggesting that substrate elasticity alone did not direct stem cell fate, but rather a network of factors such as the presence of integrins and integrin-receptor interactions was also likely at work. This highlights the importance of designing materials that are more closely related to the microenvironments found in native tissues.

Several studies have shown that the dimensionality of the substrate (2D vs 3D) has an impact on cell fate and signaling cascade. Three-dimensional scaffolds provide more precise, reproducible nano-topographical features and such nano-texturing is usually absent in 2D substrates. Certain stress mediators such as p38 and c-Jun N-terminal kinase (JNK) were significantly activated in 3D calcium phosphate scaffolds, thereby indicating that cells response to environmental signals, triggering certain signaling pathways such as MAPK cascade. This phenomenon was less evident in 2D calcium phosphate scaffolds.[65]

## Current Perspectives, Challenges and Future Directions

The substrate composition, dimensionality, mechanical properties, nanotopographical cues, elasticity, biophysical characteristics, biochemical signaling regulatory networks are some important factors that affect the differentiation lineage of MSCs. Nevertheless, the mechanisms of stem cell biology and cell-material interactions needs to be further harnessed. Standardising culture techniques and conditions for the expansion and differentiation of stem cells, and also narrowing down to a few material substrates from the plethora of material choices is a gargantuan task. The arduous, time-intensive culture process of MSC expansion can also be daunting. Kinks need to be worked out, such as the establishment of more robust culture protocols (controlled expansion and differentiation into specific lineages), effective cell delivery systems to ensure cell survival, and designing the appropriate material carriers suitable for specific clinical conditions (e.g. trauma, spinal fusion, fractures, maxillofacial reconstruction, cranial and dental applications). It is also imperative to ameliorate scale-up and characterisation efforts for effective MSC-based therapies. Particularly, growth factors that are used to hasten MSC differentiation and directly isolating MSCs from various tissue sources may not give rise to one cell-type exclusivity. In some instances, it may be more important to have an enrichment of the cell of interest. Other challenges include matching the mechanical properties of the substrates to bone and supporting angiogenesis in tissue-engineered constructs. Although harvesting MSCs from bone marrow is the gold standard, a recent study showed that adipocytes that reside in bone marrow could antagonise the haematopoietic activity in the bone marrow niche.[66] By suppressing marrow adipogenesis, haematopoietic activity may have been improved but balancing between osteogenesis and adipogenesis has to be considered as both osteoblasts and

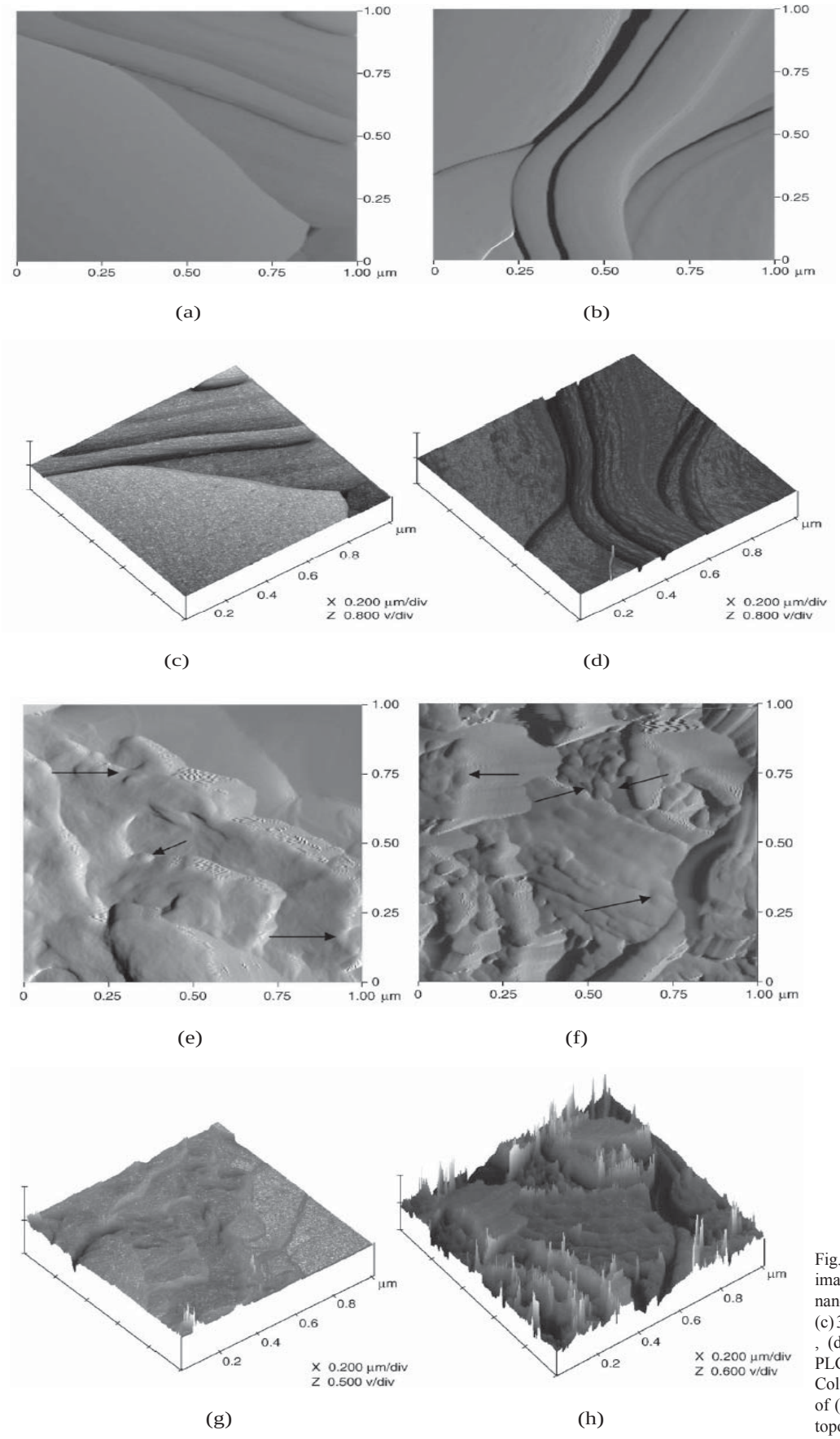



Fig. 5. Atomic force microscopy (AFM) images of nanotexturing of mineralized nanofibers.[30] (a) PLGA, (b) PLGA/Col, (c) 3D surface topography of (a) PLGA , (d) 3D surface topography of (b) PLGA/Col, (e) PLGA+HA, (f) PLGA/Col+HA, (g) 3D surface topography of (e) PLGA+HA and (h) 3D surface topography of (f) PLGA/Col+HA.

adipocytes originate from bone marrow MSCs and they have a reciprocal relationship.[67] Other reasons stymieing the clinical application of tissue-engineered constructs on a wide scale is the lack of large clinical trials. Most *in vivo* work involves animal models and there should be concerted effort in carrying out clinical trials to elucidate the true performance of tissue-engineered graft materials. These unaddressed issues call for the global push for collaborative work which aims to accelerate its clinical application.

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