Mimicking bone microenvironment: 2D and 3D *in vitro* models of human osteoblasts

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Authors declare no conflict of interest

Graphical Abstract

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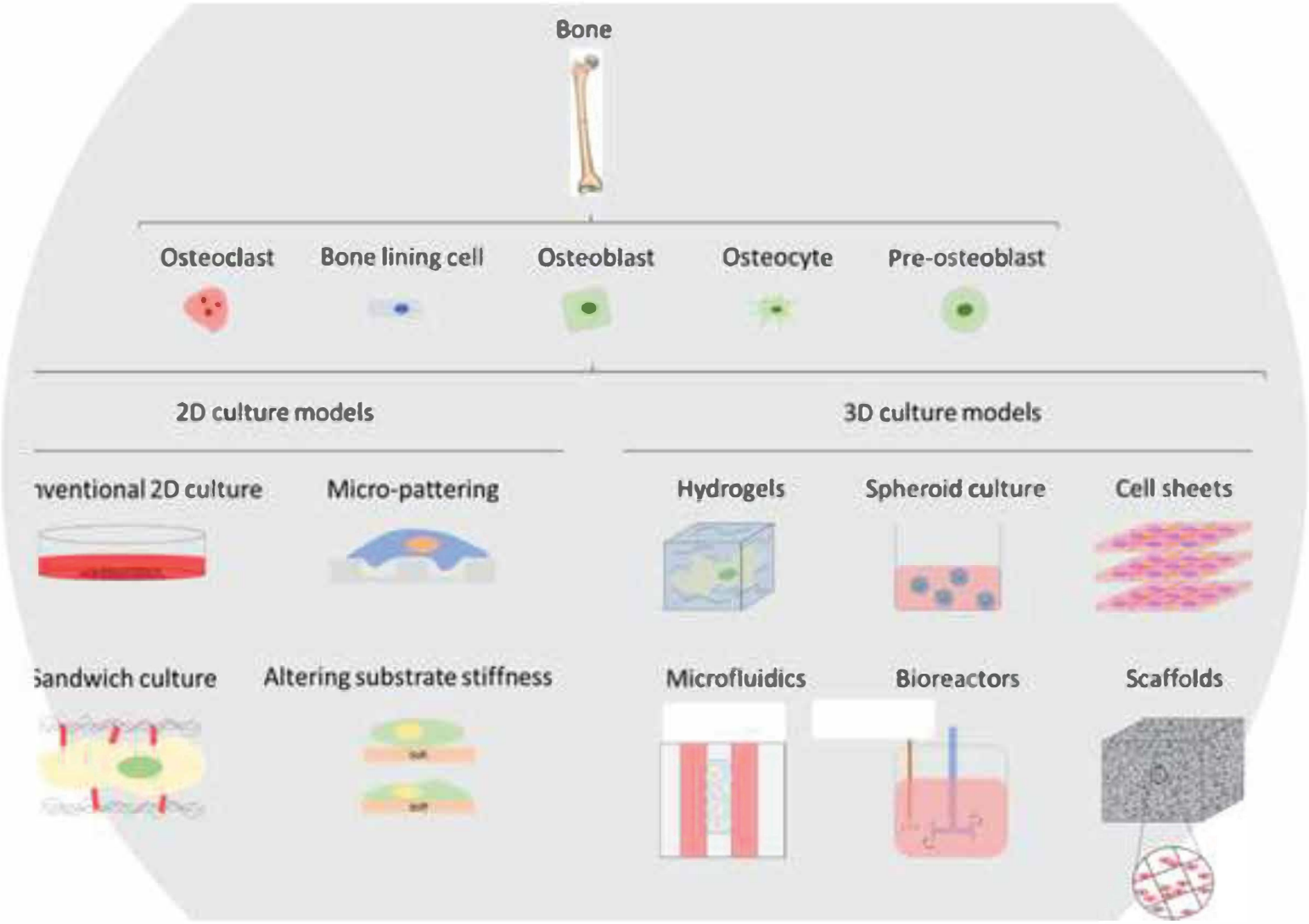

Mimicking bone microenvironment: 2D and 3D *in vitro* models of human osteoblasts

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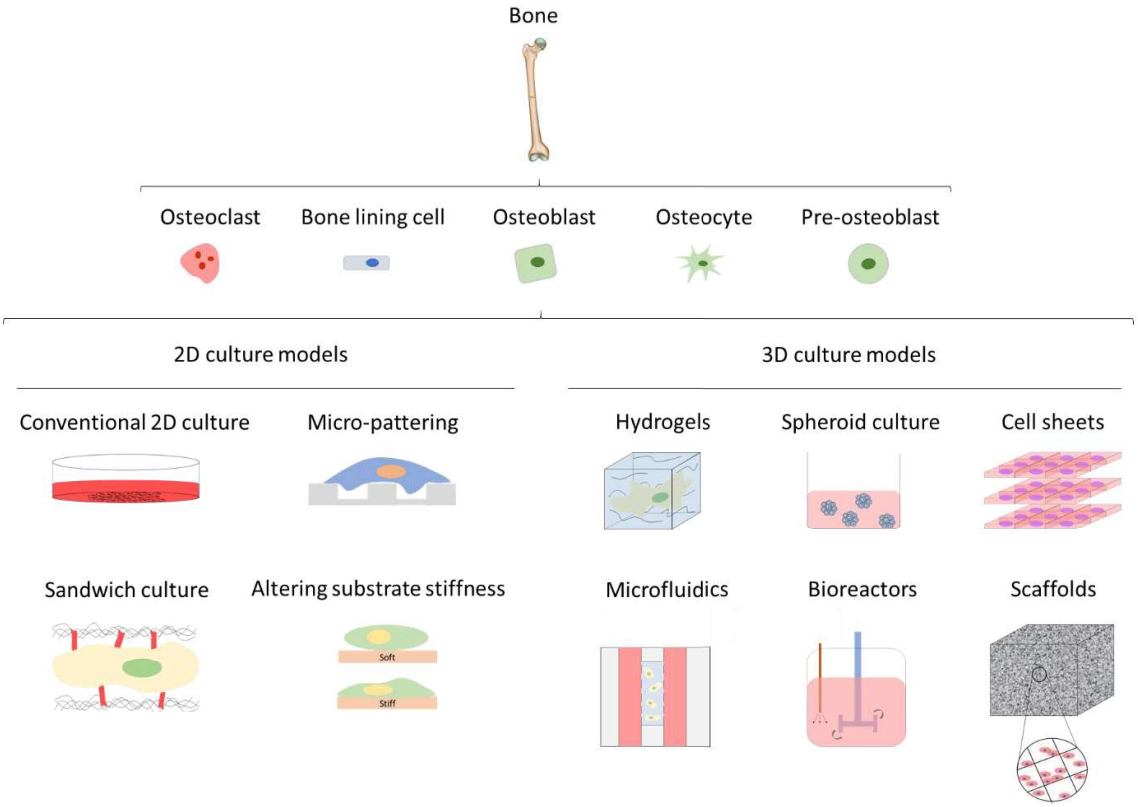
Abstract:

Understanding the *in vitro* biology and behaviour of human osteoblasts is crucial for developing research models that reproduce closely the bone structure, its functions, and the cell-cell and cell-matrix interactions that occurs *in vivo.* Mimicking bone microenvironment is challenging, but necessary, to ensure the clinical translation of novel medicines to treat more reliable different bone pathologies. Currently, bone tissue engineering is moving from 2D cell culture models such as traditional culture, sandwich culture, micro-patterning, and altered substrate stiffness, towards more complex 3D models including spheroids, scaffolds, cell sheets, hydrogels, bioreactors, and microfluidics chips. There are many different factors, such cell line type, cell culture media, substrate roughness and stiffness that need consideration when developing *in vitro* models as they affect significantly the microenvironment and hence, the final outcome of the *in vitro* assay. Advanced technologies, such as 3D bioprinting and microfluidics, have allowed the development of more complex structures, bridging the gap between *in vitro* and *in vivo* models. In this review, past and current 2D and 3D *in vitro* models for human osteoblasts will be described in detail, highlighting the culture conditions and outcomes achieved, as well as the challenges and limitations of each model, offering a widen perspective on how these models can closely mimic the bone microenvironment and for which applications have shown more successful results.

Keywords: bone regeneration, *in vitro* models, osteoblast, 3D bioprinting, microfluidics, tissue engineering, hydrogels.

Graphical abstract



1. Understanding the bone structure and remodelling

Bone is an organised, dynamic and metabolically active tissue that consists of a mineral phase made of hydroxyapatite and an organic matrix containing collagen, non-structural proteins, glycosaminoglycans and lipids [1-3]. Bone is composed by the following cells: i) osteoblasts, which are polarised, cuboidal, mononuclear cells rich in organelles and responsible for bone formation, synthesis and deposition of bone matrix proteins; ii) osteocytes, which are matured osteoblasts captured inward the bone matrix in lacunae; iii) bone lining cells, that are osteoblasts, flat and elongated, without synthetic function that cover most of the bone surfaces and iv) pre-osteoblasts, that are mesenchymal cells, precursors of osteoblasts and v) osteoclasts, which are large multinuclear cells responsible for bone resorption. Their precursors are mononuclear hematopoietic cells of the bone marrow [1, 3-6].

It is key to know the characteristics of the healthy bone when trying to mimic it through cell culture experiments. This task is challenging taking into account the high number of variables affecting its structure and mechanical properties based on age, sex, bone location, etc. Most bones present two different structures, a porous core, known as trabecular bone, and a compact shell, known as cortical bone [7, 8]. Osteoblasts are present in trabecular surfaces and the external and internal surfaces of cortical bone (endosteum and periosteum, respectively) [7]. These tissue compartments have a porosity ranging from 40-95% in the trabecular bone and 5-15% in the cortical bone, although trabecular pores are larger (~100 μm in diameter) than the cortical ones (~10 μm, but can reach 250 μm) [8, 9]. Porosity is crucial for the mechanical properties of the bones, like elastic modulus (i.e., material stiffness), toughness, elasticity, and impact energy absorption capacity of bone [8-10]. Regarding the mechanical properties, the cortical bone is anisotropic which means that the strength along the longitudinal direction is greater than along the radial and circumferential direction. For example, the human femoral cortical bone has an elastic modulus of 17,900 ± 3,900 MPa in its longitudinal direction while it is 1.7-folds lower in its transverse direction [9]. Trabecular bone is a higher porous material than the cortical bone, also with anisotropic mechanical properties which are defined primarily by its porosity. The strength of trabecular bone is greater in compression than tension and is lower in shear with a high variation in density and architecture. Within the same epiphysis, Young´s modulus can range from 10 to 3,000 MPa and the strength can also vary from 0.1-30 MPa [11]. Bone porosity and stiffness also present an age-related relationship [9, 10]. Fracture toughness decreases about 4% per decade, the strength of cortical bone under tension and compression is reduced around 2% per decade starting at the 30s and tensile ultimate strain decreases by around 10% per decade, being the highest (5% strain) at age of 20-30 years and reaching the lowest value less than 1% strain above 80 years of age [12].

Bone remodelling is a complex and necessary process that involves the replacement of the old bone with a new one to ensure skeleton integrity. Remodelling cycles takes place through three stages: i) bone resorption carried out by osteoclasts; ii) the reversal phase

characterised by osteoblast differentiation and migration to the area induced by mediators ruand signals, like glucocorticoids, oestrogens, cytokine IL-6 and osteoblast-derived PTHrP (parathyroid hormone-related protein) and iii) bone formation triggered by osteoblasts (Fig 1) which are modulated by a complex systemic and local regulation [1, 3, 13, 14].

Systemic regulation is possible via the action of hormones, like the parathyroid hormone (PTH), thyroid hormones, oestrogens, and androgens. PTH is a crucial regulator of calcium homeostasis, as when it is secreted intermittently, it induces bone formation while when its release is continuous, bone resorption is triggered. Thyroid hormones stimulate both bone formation and resorption. Oestrogens inhibit osteoclast formation and stimulate osteoblast proliferation. Therefore, bone resorption prevails over bone formation, when there is an oestrogen deficiency, which explains the prevalence of osteoporosis in menopausic women and elderly men (due to a reduction in oestrogens levels). Lastly, androgens enhance osteoblastic activity [3, 13-15].

The local regulation is orchestrated by cytokines and the OPG/RANKL/RANK system. Bone marrow cells and bone cells are both involved in this process. RANKL (receptor activator of nuclear factor-kappa β ligand, present on preosteoblastic cells) binds to RANK (receptor activator of nuclear factor-kappa β present on preosteoclastic cells) and stimulates osteoclast differentiation and proliferation, inducing bone resorption, while OPG (osteoprotegerin) inhibits RANKL. Some cytokines, like TNF-α (tumour necrosis factor-alpha) and Il-10 (interleukin-10) can increase RANKL expression, while cytokines like Il-6 (interleukin-6) induce osteoclastic bone resorption [3, 13, 14].

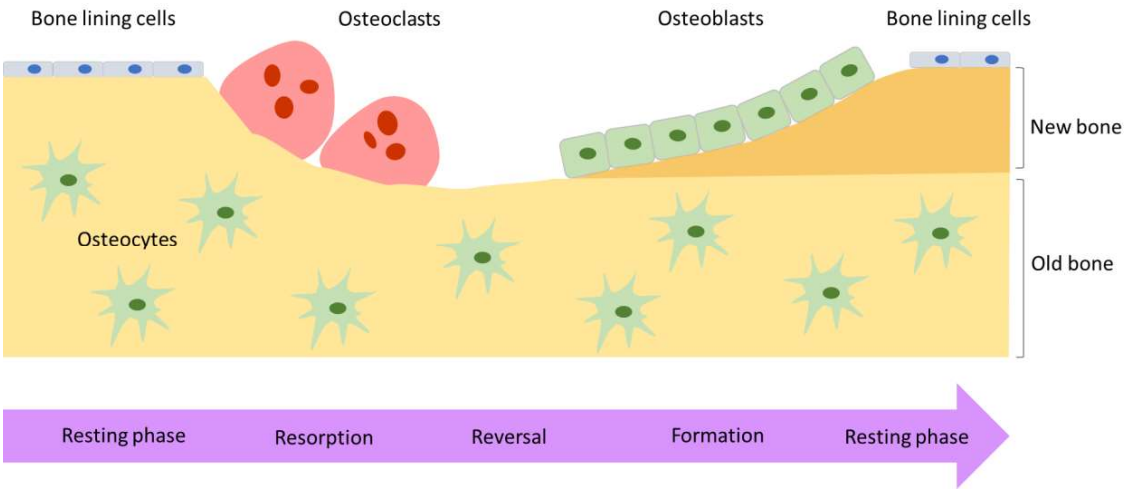


Figure 1. Schematic representation of the different phases of bone remodelling.

2. An insight into bone cell culture models

2.1. Challenges and benefits of primary *versus* secondary cell lines

As osteoblasts are the main cell in the bone, most studies have focused on the development of tissue cultures using this cell type. Nowadays, *in vitro* models of osteoblasts have been developed using primary cultures, induced osteoblasts from pluripotent stem cells, immortalised and malignant cell lines, providing valuable information from each type of cells [1, 2].

Primary cultures are cells isolated directly from a tissue, by enzymatic digestion or spontaneous outgrowth. Primary cultures have the advantage of possessing high clinical applicability compared to immortalised and malignant cell lines. Primary bone cells can be isolated from humans and animals. Cell behaviour of primary human osteoblasts is influenced by donor age, gender, and site of isolation [1, 2]. Hence, proliferation capacity of osteoblast cells is lower in older people, in postmenopausal women and in certain bones such as the femoral head. The lower proliferation capacity is related to high levels of alkaline phosphatase (ALP), and low levels of type I collagen and osteocalcin [2]. Moreover, they present a heterogenous mixture of osteoblastic cells at different stages of differentiation [1]. Animals models offer advantages for osteoblast cells isolation compared to humans. For example, the isolation of cells is not just limited to subjects with pathologies and there are more bone sites to extract cells. However, there are more differences in the biology and structure of bone between animal species [2]. Among animals, the rat is the most commonly animal used to isolate osteoblasts followed by mice, rabbits, sheeps and cattles. Osteoblast cell phenotype depends on the age, sex and origin of tissue [2].

Induced pluripotent stem (iPS) cells can be obtained by reprogramming human fibroblasts. iPS cells can differentiate to mesenchymal cells and subsequently, to osteoblasts, expressing bone-specific genes and calcified bone matrix. The differentiation can be achieved by culturing cells on matrices or surfaces containing calcium phosphate (CaP) or adding to the medium adenosine or osteoblast-specific transcription factors, like Runt-related transcription factor 2 (Runx2), Osterix, Octamer-binding transcription factor 3/4 (Oct4), and L-Myc (RXOL). This technique is efficient, low-price, and allows us to study the patient's cells, which can lead to an autologous transplant. However, environmental conditions are critical for this type of cells, which can affect reproducibility of experiments [16-18].

Osteoblast cell lines can be derived from osteosarcoma~~, a bone-forming tumour~~. These cells maintain their osteogenic capacity, expressing the phenotype before their transformation. These malignant cell lines are easy to grow, with relative genetic stability and with small changes between subcultures. Their main limitation is that typical tumour cell aberrations and genetic drift caused by heteroploidy can take place. However, they present phenotypic stability in long term cultures (>30 passages) [19, 20]. There are several human and murine osteosarcoma cell lines. The human cell lines include SAOS-2, OHS-4, HOS-TE-85, MG-63, KPD-XM, TPXM, CAL72 [1, 2]. The human osteosarcoma Saos-2 cell line shows quite a few osteoblastic features including expression of parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 receptors as well as it has high levels of alkaline-phosphatase activity. This cell line originally derived

from an 11-year-old Caucasian female with osteogenic sarcoma. The human osteosarcoma HOS-TE85 cell line, derived from a 13 year old female, is characterized by high levels of alkaline-phosphatase activity [21]. The human osteosarcoma MG-63 cell line produces high yields of interferon; this cell line originally derived from a 14 year old Caucasian male [22]. The human osteosarcoma OHS-4 cell line shows a high alkaline phosphatase activity and comes from a 14-year-old male [23]. The murine osteosarcoma cell lines include as examples K7M2 wt and MC3T3-E1 [2].

Apart from the above-mentioned cell lines, non-malignant osteoblast cell lines, known as immortalised cell lines, can be used. The immortalization of osteoblasts is carried out by transfecting a recombinant retrovirus containing the cDNA for SV40 large T antigen. Immortalised lines possess the following advantages: i) ease of maintenance; ii) production of high amounts of cells and iii) relative phenotypic stability. In contrast, these cells fail in representing the entire phenotypic spectrum of normal osteoblasts and prolonged passages lead to a progressing phenotypic heterogeneity. Human osteoblast-like (hOB) cells and human foetal osteoblast cell line (hFOB) are two common examples of immortalised osteoblast cell lines [1, 2].

2.2. Bone cell culture media

The environment is critical in any cell culture and should mimic the natural conditions for the cell type. Cells need to be able to attach (although some cells can grow in suspension) in a controlled environment in terms of temperature, oxygen, osmolarity, media composition and viscosity (liquid or semisolid with a gel-like structure created to support cell growth) (Table 1) [24]

Different types of medium can be used for osteoblasts culture, like Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F12 or α-MEM (Minimum Essential Media) [1, 2]. There are a similar proliferation and differentiation in cultures with DMEM and α-MEM. However, a significant decrease in the activity of alkaline phosphatase (ALP) (5-30 nmol/min cm^2 less), an osteoblast biomarker, and the ability to form mineral deposits have been reported when DMEM medium was used [25].

A key factor in culture medium for osteoblasts is glucose, as high levels of this compound (24-25 mM) have been reported to alter gene expression and mineralization and inhibit cell growth. The physiological concentration of glucose is around 5 mM, which is usually the concentration present in the media mentioned above [26-28].

Medium is commonly supplemented with 10% of foetal calf serum (FCS) or foetal bovine serum (FBS), and, although optional, an antibiotic drug and an antifungal compound are usually added. The most common additives included into osteoblast cell cultures, due to their ability to stimulate the osteoblastic phenotype expression, are dexamethasone (10^{-7} to 10^{-9} M), calcitriol ($1,25(OH)_2D_3$, with unclear suitable concentrations), β-glycerophosphate (βGP, 5-10 mM) and ascorbic acid (25 - 50 μg/mL). However, their concentration varies among cell types (Table 1) [1, 2]. The latter has shown to increase the levels of ALP, and hence, promoting the differentiation of osteoblasts. Only βGP and dexamethasone have demonstrated to be able to enhance the mineralised extracellular

matrix formation, while glucocorticoids, like dexamethasone, promote a detrimental effect on bone *in vivo* [29, 30].

Table 1. Osteogenic inducers added to the medium for human osteoblasts culture. Adapted from: [2].

Cell type	Most common osteogenic inducers in culture	Concentration
Primary Human Osteoblast	Ascorbic acid	50 µg/mL
	β-glycerophosphate	5-10 mM
	Dexamethasone	10-100 nM
MG-63	Ascorbic acid	50 µg/mL
	β-glycerophosphate	5-10 mM
SAOS2	Ascorbic acid	50 µg/mL
	β-glycerophosphate	0-10 mM

In human osteoblasts culture, pH is a key factor for cell development. A higher viability, proliferation and mineralization occurs at elevated pH in the range between 7,0-8,4 [31, 32]. However, there is still limited evidence about the effect of pH on *in vitro* models of human osteoblasts [31].

2.3. 2D or 3D bone cell culture models?

Cells are surrounded by a complex matrix, a net of blood vessels that are in contact with other cell types having an intricated transport system for nutrients and oxygen. Two-dimensional (2D) cell culture has been the most used technique in the last decades, but, despite their great contribution in advancing knowledge, growing evidence shows that 2D models fail to mimic *in vivo* conditions in a reproducible manner [33-38]. For example, osteoblasts, like most cell types, acquire a flat shape, which changes their natural distribution of the cytoskeleton and alters their gene expression [35, 39]. However, the 2D models are widely used as a pre-screening tool.

3D models allow cells to maintain their original shape (Fig 2), have higher stability and a longer lifespan, display a less altered genotype, and grow and interact with the environment in all three dimensions, making a good approximation to the real *in vivo* microenvironment. Also, experimental data obtained from 3D culture models are far more predictive of *in vivo* applications and thus are increasingly more utilised in research [33-37, 39-41]. 3D cell culture models have shown levels of cell organization and differentiation that cannot be achieved in 2D cell culture models. Cell requirements are different between these models, and in fact, there is no available universal 3D model easily implemented and several limitations are inherent to the type of model [34, 36, 41]. Therefore, 2D and 3D models are complementary, due to each one can provide valuable

information. Moreover, depending on the experiment or the cell type selected, the most appropriate culture model may vary, along with their specific characteristics and requirements. The different cultures models applied for culturing human osteoblasts are described in more detail in the next section [34, 39]. To get an in sight into the complexity of human bone 2D and 3D models, several representative examples have been thoroughly selected for each model.

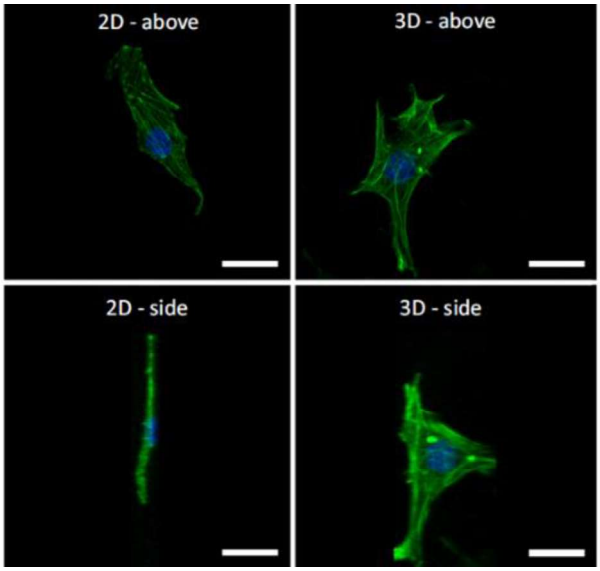


Figure 2. Effect of the physical environment in cell shape. Confocal images of a single fibroblast grown in 2D or 3D culture. The cell has been stained with phalloidin to visualize the primary structural elements of the F-actin cytoskeleton and 4',6-diamidino-2-phenylindole (DAPI) for the nucleus. The images show the shape of a typical cell when visualized from above (top panels) or the side (bottom panels). Scale bars: 10 µm. Reproduced from [35].

3. Human osteoblast *in vitro* cell culture models

3.1 2D models

First cultures of human osteoblasts were established in monolayers. 2D models have been the standard method for many decades [1, 2]. Some improvements and changes have been implemented in 2D cultures, like sandwich culture (which some authors categorise them as a 3D model) and modifications of the substrate topography and stiffness (Fig 3) [34].

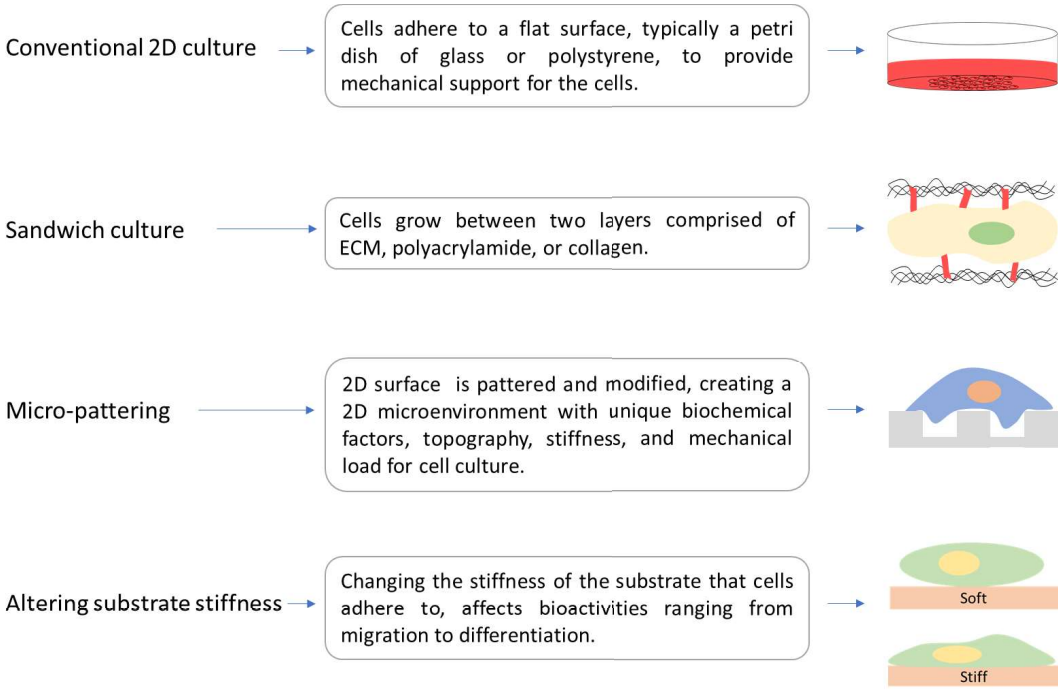


Figure 3. Schematic representation of the main characteristics of 2D cell culture methods. Key: EMC, extracellular matrix.

3.1.1 Traditional 2D cell culture models

Regular 2D cell culture consists of adhered osteoblasts to a flat surface, commonly flasks [5, 19, 42], glass, polystyrene dishes [5] or plates [43-45]. These surfaces provide mechanical support for cells which grow in monolayers. This technique allows a homogenous distribution of the components present in the medium, cell growth, and proliferation [34]. Primary cultures [5, 42, 45], malignant cell lines [19, 44], and non-malignant cell lines [43, 44] have been used in multiple studies, demonstrating that a wide range of osteoblasts cell types is suitable for this culture method. The comparison of the different cell types, culture media and supplements are shown in Table 2.

Table 2. Culture conditions applied in traditional osteoblast primary cultures and cell lines. Key: DMEM, Dulbecco's modified eagle's medium, SGM, standard growth medium, OBM, osteoblast growth and differentiation media, α-MEM, minimum essential media, L-AA, L-ascorbic acid, βGP, β-glycerophosphate, HEPES, 4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid, FCS, foetal calf serum, FBS, foetal bovine serum, LLL, low lever laser, LED, light-emitting diode.

Cell type	Culture media	Supplements	Cell seeding density	Culture surface	Aim of the study	Findings	Ref.
Primary culture (Bone explants from orthopedic surgeries)	DMEM with 10% FCS	50 µg/mL ascorbic acid and 10 mM βGP	Unspecified	25cm^2 flasks	Comparison of the influence of two spectrums of LLL and LED phototherapy on human osteoblasts proliferation and differentiation.	LLL and LED differently modulated the metabolism of human osteoblasts.	[42]
Primary culture (human cancellous mandibular bone collected from the tissue surplus as corticospongiosa bone fragments)	DMEM with 12% FBS, 1% amphotericin B (250 mg/mL), 1% penicillin 10.000 U/mL)/streptomycin (10.000 g/mL) and 1% glutamine (200mM)	1.4 mM ascorbic acid and 10 mM βGP	5.000 cells/cm^2	48 - well plates	Examination of effects of simvastatin (SV) on osteoblastic mineralization	SV induced a time and dose-dependent significant decrease of cell viability and a significant increase of mineralization	[45]
SAOS2	DMEM with 44 mM $NaHCO_3$, 2mM L-glutamine, and 10% FCS	0.2 mM ascorbic acid 2-phosphate and βGP 10 mM	1-1,5 x 10^4 cells/cm^2	75 cm^2 flasks	Comparison of the phenotypic properties when culturing cells over 100 passages	Most of the genes investigated did not show a great variation, but some genes (like decorin) exhibited remarkable differences	[19]
SAOS2	SGM composed of DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin	50 µg/mL L-AA	5000 cells/cm^2	Unspecified	Comparison of proliferation and maturation potential of three different osteoblast cell lines (SAOS2, MG-63 and MC3T3-E1) with hOBs	The cell lines presented several similarities in terms of proliferation and mineralitation compared to hOBs. However, none of them should replace primary cells studies.	[44]
MG-63		50 µg/ mL L-AA, 10 nM dexamethasone and 5 mM βGP					
hOB	OBM with 10% FCS, and 1% penicillin/streptomycin	50 µg/mL L-AA, 7.5 mM βGP, and 200 nM hydrocortisone					

hFOB	αMEM without phenol red with 2% charcoal-treated FCS	1 μM dexamethasone and 10 mM βGP	1 x 10^5 cells/well	Collagen I-coated 6-well plates	Analysis of how the mechanical stimulation effect can vary depending on the differentiation state	Differentiation showed a dependent mechanical control of osteoblasts proliferation and apoptosis	[43]

The main limitation reported to the culture of osteoblasts in monolayers is that cells grow in two dimensions, and hence, they lose their ability to distribute heterogeneously [35, 39]. In primary cultures, other cell types apart from osteoblasts can be found, like mesenchymal cells or fibroblasts, and the differentiation state may vary among cells. Hence, the latter can derive from a certain degree of heterogeneity within the culture [2, 19]. SAOS2 may behave differently depending on how they are subcultured, showing disparity among laboratories [19]. MG-63 cells do not show inhibition of proliferation by contact, so, cells in confluence keep growing uncontrollably, changing their morphology, and subcultures and experiments with this cell type should be done before a 100% confluence [20]. Although SAOS2 and MG63 share some similarities with hOB cultures and are valuable *in vitro* models, they should not replace primary cultures, due to differences in gene expression (Runx2, type I collagen (COL1), ALP, and Osteocalcin (OC)) [44]. 2D culture *in vitro* models fail to represent bone topography, as plates and flasks present planar surfaces, as well as its mechanical and chemical properties.

3.1.2 Sandwich culture

To improve 2D cell cultures and mimicking better the *in vivo* bone environment, 2D cell culture models have evolved into more complex systems. One of them is the sandwich culture technique, which some authors consider it as a 3D model rather than a 2D model. Sandwich culture consists of cells seeded between two layers of extracellular matrix, polyacrylamide or collagen. Sandwich culture has shown to be a good tool for pharmacokinetic studies, specifically with cells surrounded by complex ECMs, like hepatocytes (uptake and efflux transport) [34, 46-48], and for osteogenic differentiation . However, there is a paucity of data about its use for human osteoblasts cell culture.

3.1.3 Micro-patterning

Micro-patterning consists in the modification of substrate topography, creating a wide range of different 2D microenvironments [34]. Cellular adhesion is a critical event in cell culture and different studies have shown that surface topography, roughness and pore size affects this process, having an important role in morphology, proliferation and differentiation of bone cells [49-52]. Furthermore, it has been proven that adhesion, as well as material composition and variations of its surface, plays a key role in cell attachment to the surfaces in the first hours of culture, and usually, osteoblasts attachment is increased on grooved and rough surfaces with a Sa (Arithmetical Mean Height) > 1 and pore sizes ranging from 150 to 500 μm [50, 53]. Hence, these factors have been largely studied for osteoblasts cultures and bone implants, being their initial interactions determining for their viability [54].

The topography of a wide variety of materials has been modified for osteoblast culture. Some of them are apatite (grooves, pillars and holes) [49], titanium (electro-eroded, sandblasted, acid-etched, polished, machine-tooled and parallel or crossed grooves) [50],

poly(methyl methacrylate) (PMMA) (square prism micropillars) [52], diamond films (chemical stripe-like patterns with hydrogen and oxygen) [55] and titanium-6-aluminium-4 vanadium (Ti6-Al-4V) alloy (grooves, dots, and dimples) (Table 3 & Fig. 4)[56].

Table 3. Materials applied for micro-patterning in culture of human osteoblasts. Key: Sa, Arithmetical Mean Height, PMMA, poly(methyl methacrylate), RMS, Root Mean Square, Ti6-Al-4V, titanium-6-aluminium-4 vanadium alloy, Ra, average roughness.

Material	Micro-patterning procedure	Shape	Size		Roughness	Ref
Apaptite	Micro-molding	Grooves	2 µm (width)	2 µm (height)	Unespecified	[49]
		Pillars	2 µm, 1 µm, or 500 nm (pitch and diameter)			
		Holes	2 µm (diameter)	2 µm (depth)		
Titanium	Machine-tooling	Parallel grooves	200 µm width and 14.5 µm depth		0,7 Sa	[50]
		Cross-grooves	200 µm width and 5.5 µm depth			
	Electro-erosion	-	-		0,7 or 2,4 Sa	
	Polishing	-	-		0,7 Sa	
	Etching	-	-		0,7 Sa	
	Sandblasting	-	-		2,4 Sa	
PMMA	Standard photolithography	Square prism micropillars	8 µm of height and interpillar gaps 4, 8 and 16 µm		Unespecified	[52]
Diamond	Chemical patterning	Chemical stripe-like patterns	30 to 200 µm (width)		0.6 nm (RMS)	[55]
Ti6-Al-4V	Laser interference lithography	Grooves	10 µm and 20 µm (width)		0,5 – 2 µm (Ra)	[56]
		Dots	5 µm and 10 µm			
		Dimples				

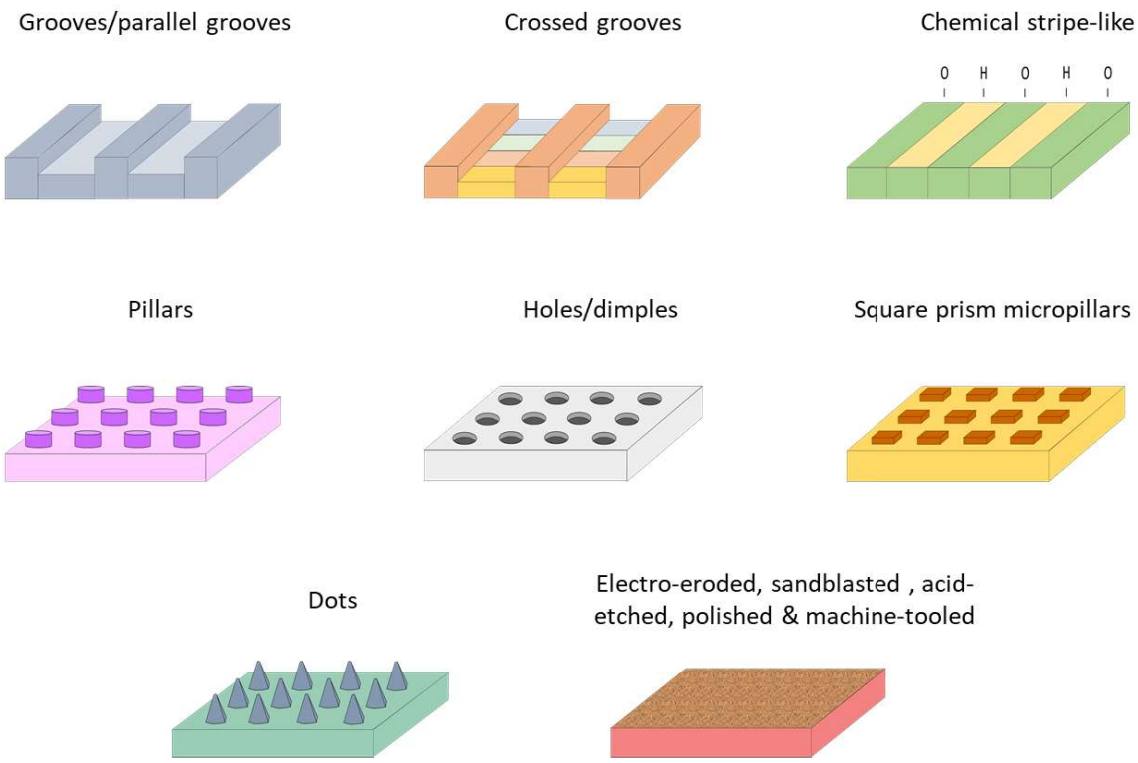


Figure 4. Schematic representation of substrate topography modification, known as micro-patterning.

These studies have shown that the modification of cell culture surface affects significantly the osteoblast morphology, adhesion and proliferation. For example, cells were highly attached in patterned apatite compared to planar apatite. Osteoblasts have a better orientation growing on grooves and were radially elongated on pillars. Moreover, cells showed a preference for patterned apatite with widths or diameters among 0.5-2 μm [49].

In pure titanium substrates, the contact of osteoblasts with substrates was more intimate on low roughness amplitude surfaces, with a Sa of 0,7, than on rougher ones (Sa = 2,4). Nevertheless, adhesion power was greater on rougher isotropic surfaces (electro-erosion, sand-blasting, or acid-etching), but lower on smoother surfaces (polishing and machine-tooling). So, osteoblasts are more sensitive to the substrate organization and morphology of the roughness than to their amplitude [50].

Applying square micropillars can help to increase cell adhesion and proliferation, compared to unpatterned surfaces. Morphological changes were observed, as osteoblasts displayed different shapes, ranging from elongated to branched morphologies. Moreover, the highest osteogenic activity was reached on surfaces with pillar dimensions and a gap width of 4 μm [52].

Chemically modified diamond films have shown that osteoblasts display a preference for O-terminated patterns. Also, when the stripes are wider than the cell size (60, 100, and 200 μm), their morphology tends to be more rounded and their proliferation is enhanced. However, the preference for O-terminated patterns does not take place when osteoblasts are cultured without serum [55].

Growing on titanium alloy, osteoblasts adhesion performance and proliferation is better when using patterned surfaces (laser interference lithography) compared to polished surfaces. Random orientations of osteoblasts have been observed in dots and dimple structures, but not in grooves, where cells are aligned in the direction of the grooves [56].

These results show that material, shape, width and roughness of the substrates are crucial factors in osteoblasts morphology, adhesion and proliferation, and these factors can vary among cell type. It would be expected that the most representative results would be obtained with surfaces that present holes or grooves, as bones *in vivo* have a porous structure and channels. Nevertheless, different topographies showed similar results, and that may suggest that for studying bone cells *in vitro* and mimicking osteoblast niche, topography shape is not as important as the size and distribution of the structures, roughness, and chemical composition of the culture surface. Bearing in mind this point, parameters must be conscientiously selected for osteoblasts culture depending on the aim of the study. This suggests that traditional 2D cell culture is a too simple model that fails to represent the microenvironment of osteoblasts. Cell source, culture medium and supplements utilised in micro-patterning osteoblast primary cultures and cell lines are summarised in Table 4.

Table 4. Culture conditions applied in micro-patterning osteoblast primary cultures and cell lines. Key: DMEM, Dulbecco's modified eagle's medium, α-MEM, minimum essential media, FBS, foetal bovine serum, BSA, bovine serum albumin.

Cell type	Culture media	Supplements	Cell seeding density	Culture surface	Aim of the study	Findings	Ref
Primary culture (trabecular bone taken from the iliac crest of young patients)	DMEM with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (bone explant culture)	None	30 explants/dish	100 mm dishes	Analysis of the efficiency of adhesion power in discriminating the long-term adhesion of human osteoblasts on titanium substrates with different morphologies	Human osteoblasts were more sensitive to the organization and morphology of the substrate roughness rather than to its amplitude	[50]
	DMEM with BSA 0.1% (isolated cells culture)	Calcitriol (1,25 $(OH)_2$ vitamin D_3) 10^{-8} M or only the vitamin D3 vehicle	2×10^4 cells/well	24 - well plates			
			Unspecified	75 cm^2 flasks			
			4×10^4 cells/well	Pure titanium substrates with different morphologies placed in 24-well plates			
MG-63	αMEM with 10% FBS, and 1% ampicillin and streptomycin sulfate	None	Unspecified	25 cm^2 flasks	Study of the MG-63 response upon surface modification of Ti-6Al-4V implant alloy	20 µm grooved structures provided better cell adhesion than the textured Ti-6Al-4V surfaces	[56]
			4×10^5 cells/mL	60 Ti-6Al-4V fragments placed in 48-well plates			
SAOS2	DMEM with 10% FBS, and 1% penicillin-streptomycin amphotericin B suspension.	None	$6{,}6 \times 10^4$ cells/cm^2	Patterned apatite scaffolds	Study the effects of the micropatterns of adhesion of SAOS2	Type and size of the apatite patterns affected the adhesion of Saos-2 cells	[49]
SAOS2	McCoy's 5A medium with FBS of various concentrations (0-15%), penicillin (20 U/ml) and streptomycin (20 µg/ml)	None	2,500 - 10,000 cells/cm^2	Chemically patterned diamond films	Examination of the adhesion and arrangement of osteoblasts on patterned diamond films	SAOS2 cells showed a preference for O-terminated patterns and their shape was affected by the width of the chemical patterns	[55]
hOB	McCoy's 5A medium with 10% FBS 1% penicillin-streptomycin, 0.5% l-glutamine, and 0.1% amphotericin B.	0.03% ascorbic acid	Unspecified	Sterile tissue culture flasks	Investigation of the effect of the micropillar features on cell morphology, attachment, proliferation, and osteogenic activity.	Micropillar enhanced osteogenic activity of hOBs on PMMA films	[52]
			3×10^3 - 3×10^4 cells/cm^2	Poly(methyl methacrylate) films placed in 12 well plates			

3.1.4 Altering substrate stiffness

Apart from substrate topography, substrate stiffness plays a key role in migration and differentiation [34]. For example, it has been proven that mesenchymal stem cells (MSCs) can reach an osteoblastic differentiation when cultured on stiffer surfaces (4,7 MPa), as osteoblasts *in vivo* grow on bone, which is a hard tissue. Hence, this parameter is of great importance to keep osteoblastic functionality [57-59]. Stiffness of a material is measured by its elastic modulus (or Young's modulus), usually expressed in megapascals (MPa), and the larger the elastic modulus, the stiffer the materials [57, 60-63]. For example, the elastic modulus of human femoral cortical bone is ≈18 MPa (longitudinal direction) and ≈7 MPa (transverse direction), and in the ECM it ranges from 100 to 1000 KPa [9, 64].

Some of the materials employed for testing the effect of substrate stiffness on osteoblasts are methyl acrylate/methyl methacrylate (MA/MMA) polymer [57], electrospun collagen (EC) and electrospun gelatin (EG) [60], methacrylate and acrylate-based networks (poly(ethylene glycol), dimethacrylate (PEGDMA), diethylene glycol dimethacrylate (DEGDMA)) and 2-hydroxyethyl methacrylate (2HEMA) with PEGDMA or DEGDMA [61], uncompressed collagen gel (UC), bioglass incorporated uncompressed collagen gel (UC + BG), plastically compressed collagen gel (PC), and bioglass incorporated plastically compressed collagen gel (PC + BG). [62], and collagen type I and poly(lactic acid-co-glycolic acid) (PLGA) [63] (Table 5).

MA/MMA polymers with different stiffness can modulate certain hOBs behaviours. Cells were more widespread on less stiff surfaces (0.8 ± 0.1 MPa), resulting in a higher number of cells, greater ITGA1/5 (integrin subunit α1) and ITGB1 (integrin subunit beta 1) levels and lower OC, ITGB3 osteoprotegerin expression and ALP activity. On the contrary, cells growing on stiffer surfaces (309.9 ± 6.5 MPa) expressed lower levels of ITGB1 and higher levels of ITGB3. No significant morphological changes were observed and actually, hOB expression levels of osteoblastic genes only increased on stiffer surfaces (223.7 ± 31.5 and 309.9 ± 6.5 MPa). Bearing in mind these results, substrate stiffness play an important role in osteoblastic differentiation, like happens *in vivo*, which is increased with higher stiffness [57].

There was no significant difference in cell adhesion or proliferation between EC and EG matrices. However, cells grown on EC matrix showed greater expression of certain osteoblasts biomarkers, like OPN (osteopontin), ALP and OC (osteocalcin), an increase in the phosphorylation levels of Y397-FAK (focal adhesion kinase, which induces osteoblastic differentiation), ERK1/2 (extracellular signal-regulated kinase, that regulates osteoblastic maturation) and BSP (bone sialoprotein, a bone-specific extracellular matrix protein). As EC is stiffer than EG (94.296 ± 15.18 MPa vs 71.886 ± 21.10 MPa), the results obtained suggest that stiffer materials enhanced osteoblastic differentiation [60].

Copolymers with different compositions of PEGDMA, DEGMA and 2HEMA, were tested for culturing osteoblasts. A higher differentiation level of MG-63 cells was observed on PEGDMA-DEGMA surface compared to 2HEMA-PEGDMA, showing elevated levels of OC, OPG, and VEGF-A. Moreover, these values were observed with

the copolymer consisting of 10%PEGDMA:90%DEGDMA, being the stiffest combination between these two materials (with unespecified elastic modulus). This suggests that copolymer stiffness as well as chemistry are both crucial factors that regulate osteoblast differentiation [61].

Collagen densification (PC gels) can increase mineralization (resulting in more mineralized nodules) and ALP activity to a greater extent. These results are better than those obtained with UC and UC + BG gels, indicating that an increase in surface stiffness by collagen densification is better for osteoblasts differentiation rather than bioglass. Moreover, collagen densification via plastic compression also enhanced osteoconductivity [62].

PLGA (7,000 MPa) is stiffer than collagen (366.2 MPa (uncrosslinked) or 421.9 MPa (crosslinked)). When SAOS2 cells grow on PLGA substrates, the nuclear deformation is higher, while proliferation is lower compared to collagen surfaces. However, ALP production was similar in both materials. Thus, it is not clear which factor (surface chemistry or stiffness) plays a more significant role in osteoblast growth and differentiation. Besides, the impact of a single factor on cell growth is difficult to investigate as many aligned factors are interacting and playing a key role in the cell growth outcome [63].

In summary, these results confirm that stiffness influences proliferation and gene expression in osteoblasts culture. A greater osteogenic differentiation occurred in stiffer surfaces, although the values of elastic modulus were far greater than the ones found *in vivo*. Nevertheless, values vary among materials, which demonstrates that surface chemistry is also a crucial factor. More studies need to be done to identify which cell parameters are more influenced by surface chemistry and stiffness. Cell source, culture medium and supplements utilised to investigate the effect on substrate stiffness are summarised in Table 5.

Table 5. Culture conditions applied in osteoblast primary cultures and cell lines when altering substrate stiffness. Key: DMEM, Dulbecco's modified eagle's medium, MEM, minimum essential media, RPMI, Roswell Park Memorial Institute, EC, electrospun collagen, EG, electrospun gelatin, PEGDMA, poly(ethylene glycol), DEGMA, diethylene glycol dimethacrylate, 2HEMA, 2-hydroxyethyl methacrylate, UC, uncompressed collagen gel, BG, bioglass, PC, plastically compressed collagen gel, PLGA, poly(lactic acid-co-glycolic acid, β-glycerophosphate, MA, methyl acrylate, MMA, methyl methacrylate, FBS, foetal bovine serum, ROCK, Rho kinase, FAK focal adhesion kinase.

Cell type	Culture media	Supplements	Cell seeding density	Culture surface	Material type	Surface Stiffness	Aim of the study	Findings	Ref
MG-63	MEM with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin	Ascorbic acid (50 mg/ml), 10 mM ß-GP	5 x 10^4 cells/well	EC matrices placed on 24-well tissue culture dishes	Synthetic	94.29 ± 15.18 MPa	Study the effect of matrix stiffness on intracellular signalling	Osteogenic differentiation of MG63 osteoblast-like cells on EC and EG is influenced by matrix stiffness and via ROCKFAK- ERK1/2.	[60]
				EG matrices placed on 24-well tissue culture dishes		71.89 ± 21.10 MPa			
MG-63	DMEM, with 10% FBS and 1% penicillin-streptomycin	None	2 x 10^4 cells/cm^2	Tissue culture polystyrene and polymer discs	Synthetic	Unspecified	Evaluation of how the surface stiffness and chemistry affect the *in vitro* response of human MG63	Copolymer chemistry is the primary regulator of osteoblasts differentiation	[61]
				Polymer discs of PEGDMA-DEGMA, 2HEMA-PEGDMA and 2HEMA-DEGMA		60 - 850 MPa			
SAOS2	RPMI medium supplemented with 15% FBS, 1% L-glutamine, and 1% penicillin- streptomycin	None	3 x 10^4 cells/cm^2	UC (placed on 48-well plates)	Natural	Unspecified	Analysis of the effects of collagen densification and bioglass incorporation to understand the interplay between collagen packing density and presence of bioglass on cell-mediated mineralization	Collagen densification via plastic compression improves the osteoconductivity of collagen gels	[62]
				UC + BG (placed on 48-well plates)		≈ 0,05 MPa			
				PC (placed on 48-well plates)					
				PC + BG (placed on 48-well plates)		≈ 1,1 MPa			
SAOS2	RPMI 1640 medium with 10% FBS and 100 µg/mL streptomycin	None	8,5 x 10^3 cells/film	Collagen type I	Natural	366.2 MPa (uncrosslinked) or 421.9 MPa (crosslinked)	Investigation of how surface chemistry and topography can be engineered to change the conformation of cell cytoplasm and nucleus	It is not concluded which factor (surface chemistry or stiffness) is more significant on osteoblast growth and differentiation	[63]
				PLGA		7000 MPa			

hOB	DMEM with 10% FBS and 1% penicillin-streptomycin	None	5 x 10^3 or 10^4 cells/cm^2	Copolymer of MA and MMA crosslinked with 10% PEGDMA	Synthetic	0.1 - 310 MPa	Examination of the role of stiffness in MSC differentiation to two closely related cell phenotypes: osteoblasts and chondrocytes	Substrate stiffness is an important mediator of osteoblastic differentiation, and integrin β1 plays a pivotal role in this process	[57]

3.2 3D models

Cells, *in vivo*, are either surrounded by an ECM or in direct contact with other cells from the same or different lineage. Their activities respond to the stimuli of the microenvironment in which cells are growing. Despite advances in 2D cell culture methods, they fail to represent these complex interactions. Owing to these limitations, in the last decade, the development of 3D culture models has widened the possibilities for mimicking *in vivo* conditions more precisely. It has been proven that cells, cultured in a 3D environment, have different behaviours from cells growing in monolayers. A wide variety of 3D models have been developed, like spheroids, cell sheets, scaffolds, hydrogels, bioreactors, and microfluidics, and those applied in osteoblasts culture will be described in the next sections in more detail (Fig 5) [34, 65].

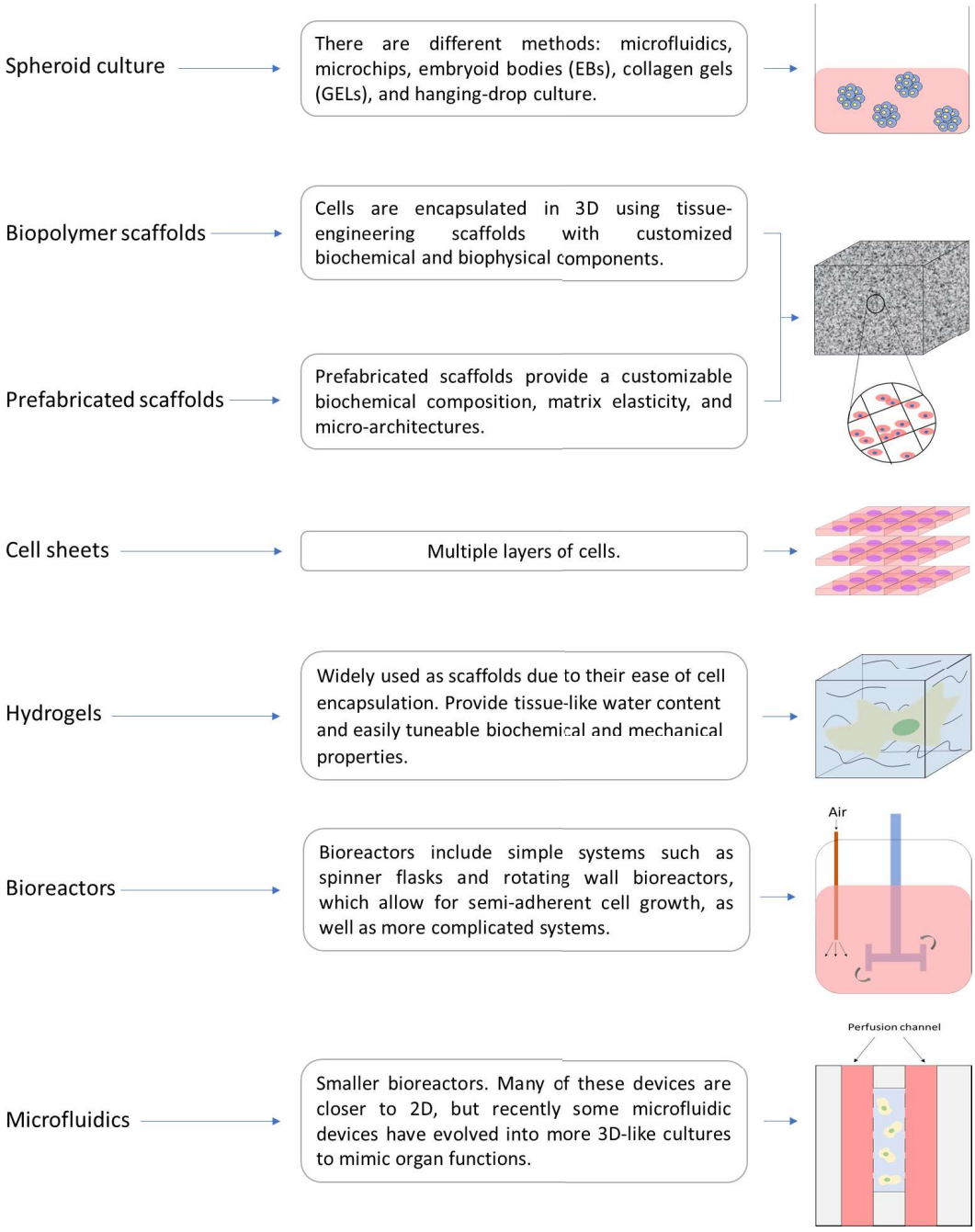


Figure 5. Schematic representation of the main characteristics of 3D cell culture models.

3.2.1 Spheroid cultures

3D models based on cell spheroids have allowed studying cell-cell and cell-matrix interactions, achieving a closer representation to *in vivo* conditions than 2D models (induction of cellular polarity and enhancement of cell-cell and cell-ECM adhesion/ signaling) [65, 66]. Nevertheless, they still are far from ideal due to the incapacity of analysing confluence, the irregular distribution of oxygen (with a lower concentration in the core), and low reproducibility. The osteoblasts viability *in vivo* is contact-dependent with the ECM, and without this interaction, programmed cell death is induced (anoikis). Based on this fact, to avoid cell death, spheroids formation has to occur in an optimum environment and as quickly as possible, as the longer it takes to construct the spheroid, the higher the chances that the spheroid structure is significantly altered [67].

Different strategies have been employed to develop a spheroid cell culture that allows studying bone cell interactions with other cells and ECM. Amongst them, the most successful techniques are the following: i) microfluidics and microchips [68], ii) embryoid bodies (aggregates of pluripotent stem cells), iii) collagen gels [69], iv) liquid overlay technique (LOT), based on the addition of a non-adherent material that avoids cell growth on the culture surface (plate, dish or flask) [66-71], v) increase of viscosity in the media, for example, by adding carboxymethyl cellulose (CC), which avoids cell deposition on the culture surface [70] and vi) hanging-drop culture (HDC) in which a drop of fluid, containing the cells, hangs from a surface [70] (Fig 6) [34].

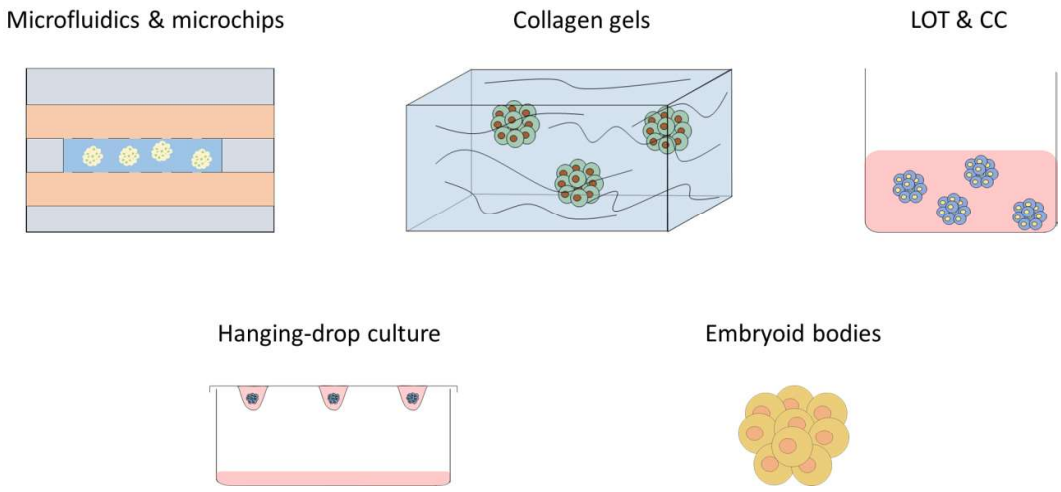


Figure 6. Schematic representation of the most commonly used spheroid cell culture techniques.

Spheroids of SAOS2 cells (S-C) have been created using the LOT and then, cultured in microreactors under different conditions: i) loaded with AMV1 (artificial matrix vesicles) with TNAP (tissue-nonspecific alkaline phosphatase) inside and attached to the membrane, ii) loaded with AMV2 (artificial matrix vesicles with TNAP only inside the medium), iii) MV (matrix vesicles) and iv) spheres of alginate particles (M^E). Under each circumstance, different cospheroids are formed: i) S-M^{AMV} (SAOS2 with AMV1 or AMV2), ii) S-M^{MV} (SAOS2 with MV) and iii) S-M^E (SAOS2 with alginate particles). In S-M^E, SAOS2 cells viability and biomineralization was increased compared to S-C. However, the greatest mineral content was observed in S-M^{MV} (increase in mineralized matrix rate of 12.3 ± 0.4% per day, and 20-50 % more calcium on day 14), in comparison with S-C and S-M^E (increase in mineralized matrix rate of 8.3 ± 0.5 % and 10.5 ± 0.2 % per day). These results demonstrate that spheroids containing only SAOS2 cells have lower viability and mineralization than cospheroids of osteoblasts that include other components [68].

SAOS2 spheroids were also formed using LOT, but in this case, the culture surface (U-bottom plates) was coated with sterile ultrapure agarose. The purpose of this research was to investigate the toxicity of titanium dioxide nanoparticles (TiO_2 NPs). Cell viability was not modified except with higher concentrations of TiO_2 NPs that resulted in increased collagen deposition, pro-inflammatory cytokines, chemokines, and growth factor secretion, affecting the cell cycle [66].

Cospheres of hOB and human umbilical vein endothelial cells (HUVECs) were created with LOT and were seeded in collagen gels to study the angiogenesis process *in vitro*. Osteoblastic cells were mainly located in the core while endothelial cells were in the shell. HUVEC spheroids possessed the capacity of forming tube-like structures under angiogenic stimulation with VEGF (vascular endothelial growth factor). However, this capacity was inhibited in HUVEC/hOB cospheroids, showing the hOB cell ability to suppress the angiogenesis process. Besides, cellular protrusions were disorganised and more predominant in hOB and HUVEC/hOB spheroids compared to HUVEC spheroids [69].

To study material-cell and cell-cell interactions [67], and cell contact-dependent gene regulation [71], hOB spheroids and cospheroids were cultured in suspension. Agar coating was used for LOT, avoiding cell adhesion on the culture surface (round-bottom plates), and inducing cell aggregation. Experimental times of hOB spheroids formation were higher compared to MC3T3-E1 cells (murine preosteoblasts) and showed a better uniformity and low multiplicity at densities in the range of 30,000-50,000 cells. More stable spheroids were obtained at higher cell densities where most cells in the aggregate core were viable. These results suggest that spheroids formation is influenced by the cell type but also by the initial cell density. Moreover, spheroids maintained good stability and viability when testing metallic and polymer-based biomaterials [67]. For the formation of HUVEC and hOB spheroids and cospheroids, cells were seeded in non-adherent round-bottom plates. Individual hOB spheroids led to significant alterations in

gene expression compared to 2D cultures, shown by the upregulation of angiopoietin-2. Gene expression was also altered in cospheroids (HUVEC and hOB cells), showing lower expression of VEGF and a higher expression of ALP in hOBs. Hence, hOB gene expression is contact dependent [71].

The hanging drop technique, CC, and LOT were compared for the formation of mono- and cospheroids of hOB fibroblasts and endothelial cells. The best spheroid reproducibility was achieved by LOT, with a yield of 60-100% for mono-spheroids and 100% for cospheroids. Varying the number of initial cells allows controlling the spheroid size. Reproducible spheroids could not be generated with HDC. Over 5000 hOB cells were needed to form spheroids with CC, although their shape was more ellipsoidal. HDMEC (human dermal microvascular endothelial cells) and hOB cospheroids presented the highest diameter and had a better organization and defined morphology than mono-spheroids [70].

In summary, the 3D culture of osteoblasts as spheroids has shown that gene expression, viability, and morphology are contact-dependent and vary between mono-spheroids and cospheroids, having a significant impact on cell function. One of the main limitations of spheroids is that the porosity and mechanical properties cannot be studied due to the type of culture as cells are suspended in the medium. Culture conditions utilised for osteoblast mono- and cospheroids are summarised in Table 6.

Table 6. Culture conditions applied in osteoblast primary cultures and cell lines when cultured as spheroids. Key: DMEM, Dulbecco's modified eagle's medium, ECGM, endothelial cell growth medium, ECBM, endothelial cell basal medium, LOT, liquid overlay technique, HDT, hanging drop technique, CC, carboxymethyl cellulose, CCT, carboxymethyl cellulose technique, FCS, foetal calf serum, FBS, foetal bovine serum, βGP, β-glycerophosphate, TiO_2 NPs, titanium dioxide nanoparticles.

Cell type	Culture media	Supplements	Cell seeding density	Culture surface	Aim of the study	Findings	Ref
SAOS2	DMEM with 10% FBS and 1% penicillin/streptomycin	None	1, 2, 3 and 5 x 10^4 cells/well (spheroids)	25 or 75 cm^2 flasks (monolayers) and U-bottom plates coated with ultrapure agarose (spheroids)	Analysis of the toxicity of titanium dioxide nanoparticles in the human osteoblast-like spheroid model	TiO_2 NPs may have a therapeutic potential to prevent or reverse bone resorption	[66]
SAOS2	McCoy's 5A cell media with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine	Ascorbic acid (50 µg/ml) and βGP (7.5 mM)	1,9 or 3 x 10^6 cells/flask (monolayers) and 10^4 cells/well (spheroids)	75 cm^2 flasks (monolayers) and 96-well spheroid microplates with ultralow attachment surface coating	Assessment of the interaction of SaOS-2 cells and microreactors	Spheroids containing only SAOS2 cells have lower viability and mineralization than cospheroids of osteoblasts that include other components	[68]
hOB	DMEM with 15% FCS (2D monoculture)	None	Unspecified	75 cm^2 flasks	Investigation of the performance of different spheroid culture techniques to form mono- and co-culture spheroids.	The most suitable technique for culturing spheroids is cell type-dependent	[70]
	Medium with 0.1% or 0.25% CC (HDT)		500, 5000 and 20000 cells/well	100 mm dishes			
	Medium with 0.1, 0.25 or 0.5% of CC (CCT)			round-bottomed 96-well plates			
	DMEM with 15% FCS (LOT)		500, 2500, 5000, 10 000, 20 000, 50 000 and 100000 cells/well	96-well plates			
hOB	Medium 199 with Earle's salt, 10% FCS, 1% L-glutamine, and 1% penicillin–streptomycin (2D monoculture)	None	Unspecified	75 cm^2 tissue culture dishes.	Assessment of the interactions between human endothelial cells (hECs) and hOBs *in vitro*	Formation of HUVECs and hOBs heterogeneous cospheroids is feasible with a distinct spatial organization	[69]

	ECBM with 20% methocell (LOT coculture spheroids)						
	0.25 mL of ECBM containing 10% FCS (culture in collagen gel)	VEGF-165 (25 ng/mL) and fibroblast growth factor (25 ng/mL)		Non-adherent 96-well plates			
hOB	DMEM with 10% FBS, 2mM L-glutamine, 0.1% non-essential amino acids, and 1% streptomycin-penicillin (2D monoculture)	None	Unespecified	Unspecified	Evaluation of material-cell and cell-cell interactions	Spheroids formation is influenced by cell type but also by the initial cell density. Spheroids maintain good stability and viability in the presence of metallic and polymer-based biomaterials	[67]
	DMEM with 10% FBS (spheroid culture)		5, 10, 20, 30, 40, 50, 75, 90, and 110 × 10^3 cells/well	Round-bottomed 96-microwell plates			
hOB	Medium 199 with Earle's salt, 10% FCS, 1% l-glutamine, and 1% penicillin/streptomycin (monoculture)	None	Unespecified	Unespecified	Study cell contact-dependent gene regulation between endothelial cells and osteoblasts	Bi-directional gene regulation mechanisms between EC and OB are established playing a critical role during osteoblasts differentiation	[71]
	ECGM with 10% FCS and 0.25% (w/v) carboxymethylcellulose (coculture)		500 cells/well	Nonadherent round-bottom 96-well plates			

3.2.2 Scaffolds

Bone tissue engineering aims to achieve an optimal bone regeneration, where bone structure (including every bone cell types) and other related tissues (like blood vessels or nerves) need to be reconstructed [72, 73]. Autografts or allografts, employing patient or donor bone cells respectively, are one of the most common transplants. However, these techniques have some drawbacks, like the limited amount of bone cells that can be extracted from the same person or the development of an immune response towards the allografts [72-76]. To overcome that, the use of synthetic materials, to create scaffolds, has emerged as a new approach to bone regeneration [72-75].

Scaffolds create a complex 3D microenvironment similar to bone structure (porosity and mechanical properties), existing cell-cell, and cell-matrix interactions. A wide variety of materials (metals, polymers, and natural materials) and techniques (electro-spinning, 3D printing, leaching (Fig. 7)), have been developed and tested for their fabrication [72-75, 77]. Different scaffold models have been applied for human osteoblasts cultures, and some of them will be described in the next section.

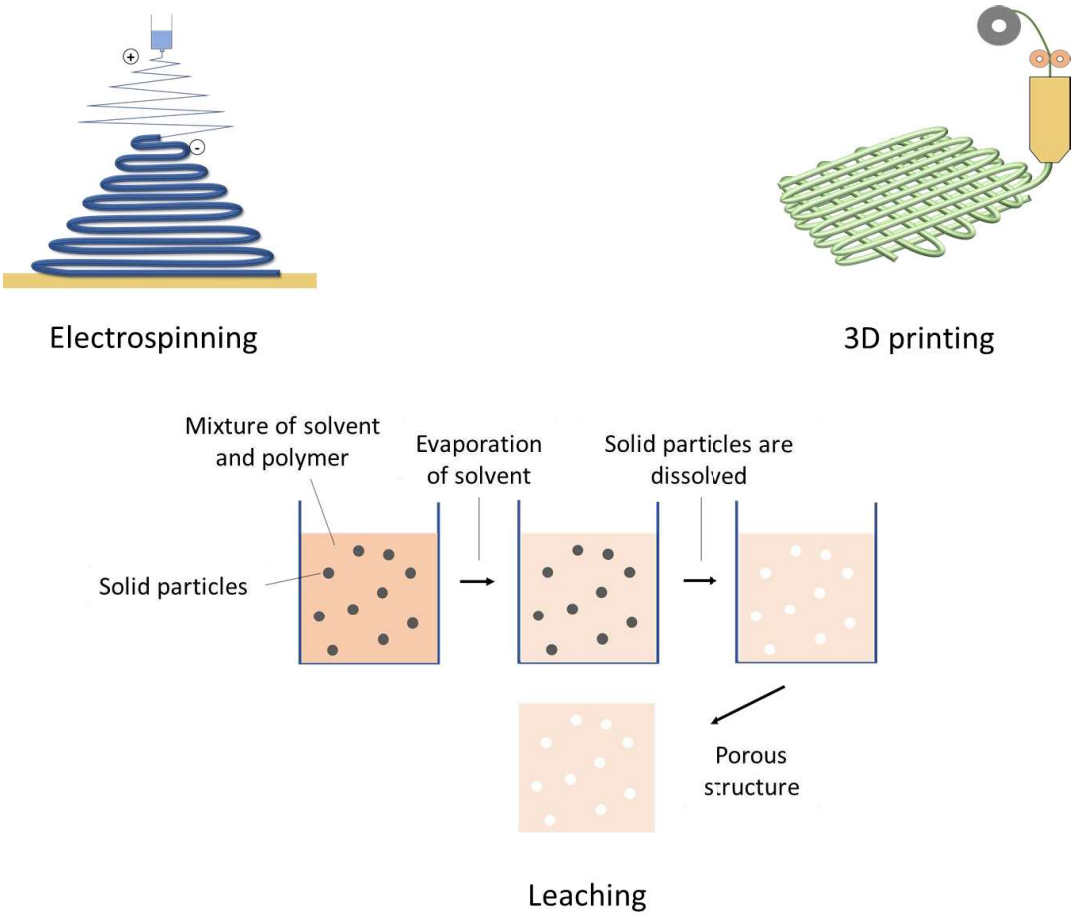


Figure 7. Schematic representation of several scaffold fabrication techniques.

Natural materials, like collagen or chitosan, are optimal substrates as most of them are biocompatible and biodegradable. Keogh et al. [78] created a collagen scaffold by lyophilisation (with a porosity of 99,5% and pore diameter of 96 μm), where hFOB cells were seeded. The mixture was previously degassed under vacuum, and then lyophilised at -40ºC followed by a de-hydro thermal crosslinking (to obtain cross-linked scaffolds) at 105ºC. Osteoblasts reached a uniform attachment, infiltration, and distribution, as well as a differentiated phenotype and mineralised bone formation. Zhang et al. [79] developed three types of scaffolds (with a pore size of 100 μm) combining chitosan as a natural product with bioceramics consisting of hydroxyapatite (HAP): HA scaffolds nesting chitosan sponges (HC1), chitosan scaffolds incorporating hydroxyapatite powder (HC2, chitosan/HA/glass = 90/10/0 mol%) and calcium phosphate glass (HC3, chitosan/HA/glass = 90/5/5 mol%) for culturing MG63 cells. HC1 scaffolds exhibited higher levels of ALP and OC in comparison with HC2 and tissue culture plates, while H3 scaffolds made of calcium phosphate glass increased ALP and OC production. These scaffolds are a promising tool for bone engineering, but further human *in vivo* studies are required. However, they only focus on mimicking the porosity of the bone, as mechanical properties of the scaffolds are not analysed.

Since the first bioactive glass was synthesised in the early 1970s, these materials have been widley studied for bone tissue engineering, due to their good osteointegration, stimulation of osteogenesis and resorption [80, 81]. Gentile et al. [82] developed chitosan/gelatin (POL) scaffolds with different concentrations of CEL2 (CEL2/POL 0/100; 40/60; 70/30 wt %/wt), a bioactive glass. These scaffolds showed different pore sizes (from 179 ± 5 μm for CEL2/POL 0/100 to 136 ± 5 μm for CEL2/POL 70/30) and the compressive modulus increased when the highest amount of CEL2 was used (2.1 ± 0.1 MPa for CEL2/POL70/30). Biocompatibility was tested with MG-63 cells, which presented an optimal viability and metabolic activity in all of them. To analyse the role of an increase of bone morphogenetic proteins (BMPs) in MG-63 cells, glass-ceramic scaffolds (CEL2) were employed by Muzio et al. [83]. Pore size of the scaffolds ranged from 200 to 800 μm. Shock-wave was applied to the cells for increasing BMPs levels. This techinque, combined with the developed scaffolds, allowed an increase in osteogenesis resulting in higher concentrations of ALP and OC. Moreover, a viability nearly 100% was also achieved. Despite the positive results obtained with these materials, it is still quite challenging to obtain an optimal balance between porosity and mechanical properties of the scaffolds. New trends are focusing on the combination of different materials, the modification of surface chemistry and the design of hierarchical systems including porous nanoparticles [80, 81].

Ceramic materials have been also commonly used for scaffold formation, like HAP (present in human bone) and tricalcium phosphate (TCP). Tarafder et al. [84] designed TCP scaffolds using microwave sintering (consisting of heat hardening of the scaffold) and 3D printing, characterised by the deposition of powdered material in layers followed by the selective binding of the powder by ink-jet printing using a binding material, followed by the removal of the unbound powder. A nanostructure was engineered with controlled pore sizes, 500 μm, 750 μm, and 1000 μm, and a mechanical strength ranging

from 6.6 - 10.9 MPa. hFOB were cultured in these scaffolds. All pore sizes enhanced bone formation, with the cell density being higher in smaller pore sizes. Feng et al. [85] incorporated HAP whiskers to a calcium silicate matrix, creating a scaffold with a pore size of 0.5~0.8 mm with improved strength (increased to 20 wt.%) and fracture resistance (with 30 wt.% of HA whiskers). There was not transgranular fracture leading to an optimal spreading and proliferation of MG-63 cells on these scaffolds. TCD and HAP scaffolds with well-defined and regular dimensions (cubes of 5 mm x 5 mm x 5mm with a pore size of 0.5 mm) were also constructed using 3D printing (inkjet printing) [86]. This technique allowed creating personalised scaffolds adapted to the patient's needs. HAP scaffolds showed good biocompatibility and a higher number of primary human osteoblasts. In conclusion, the use of ceramic materials has shown successful results in bone engineering due to their porous structure and the improvement of the scaffolds' mechanical strength [84-86]. However, their fragility and slow degradation are their main limitations for their clinical translation [74].

Metals have been widely used in bone replacements due to their excellent mechanical properties, being titanium the most popular one [74, 77]. A titanium alloy (Ti-6Al-4V) has been used to create different scaffolds, through selective laser melting (SLM) or electron beam melting (EBM), in inert gas or vacuum atmosphere by layerwise melting of the loose powder particles. The scaffolds had different porosity (51-76%), pore size (400-1000 μm), and structure (cubic, pyramidal or diagonal). Cell activity and matrix formation of human primary osteoblasts were enhanced in all the scaffolds. Nevertheless, the one that showed the best proliferation and migration levels was the scaffold manufactured by SLM possessing the highest porosity, smallest pore size, and pyramidal structure [87]. The same titanium alloy was selected by Wieding et al. [88], also fabricated with SLM, to obtain a defined pore geometry and porosity (around 70%). The comprehensive strength range from 140 to 220 MPa and the elastic modulus from 3.7 to 6.7 GPa, which are far from the values described for healthy human bone. Proliferation and spreading of primary human osteoblasts were successful in these scaffolds. Pure titanium scaffolds (pore sizes of 200 μm and 500 μm, and an elastic modulus of 42.7 and 13.3 GPa), fabricated with SLM, were chemically-treated with HF/HNO_3 to remove unmelted powder particles [89]. The chemical treatment did not impact negatively on MG-63 cell proliferation and differentiation. Moreover, these treated scaffolds increased osteoblast colonization. Although metallic materials are commonly used in bone engineering due to their numerous benefits, they also present some drawbacks. For example, metals fail to support osseointegration *in vivo*, interfere with bone remodelling and tend to get encapsulated by fibrous tissue. These drawbacks may be due to the high values of stiffness and mechanical strength of these materials. To overcome these limitations, metals are combined with other materials, by direct mixing or by the application of coatings [74, 77].

Since the late 1980s, a range of polymers has shown great potential due to their mechanical properties and their biocompatibility and biodegradability characteristics, such as polycaprolactone (PCL), polylactic – co- glycolic acid (PLGA) and polylactic

acid (PLA) [74, 77]. Kyriakidou et al. [90] designed a rotary cell culture, co-seeding MG-63 cells, and HUVEC cells onto a PCL scaffold (pore size of 200 mm and elastic modulus of 134.6 ± 8.5) which was fabricated with a bio-plotter dispensing machine, where PCL pellets were placed into a syringe, heated and extruded forming PCL fibers. Cell adhesion of both cell types was optimal. Osteoblastic differentiation was inhibited by endothelial cells, but they enhanced osteoblasts growth, leading to a vascularised-like culture.
Polymeric electrospun scaffolds were designed by Aragon et al. [91]. The polymers selected were PCL and polycaprolactone/polyvinyl acetate (PCL/PVAc), decorated with poly(lactic-co-glycolic acid) [PLGA] particles and loaded with bone morphogenetic protein 2 (BMP2). They presented a porosity of 60-64%, with pore sizes ranging from 0.2 to 2.5 μm which are similar to cortical bone pores. hOBs were seeded and showed optimal cell growth and proliferation, as well as good levels of osteogenic and osteoconductive markers. Better results were found in scaffolds loaded with BMP2, which suggests that the addition of BMP2 or other related proteins to the scaffolds have a great potential for improving bone remodelling and hence, their clinical application. Proliferation and differentiation of hFOB cells in PLGA scaffolds were evaluated by Ge at al. [92]. Despite PLGA scaffolds showed similar mechanical properties to trabecular bone (50% of porosity and young modulus of 7.8 ± 3.1MPa and 77.2 ± 10.8 MPa), they were weaker in terms of mechanical strength. hFOBs exhibited good proliferation and viability (95% ± 6%, N=6, at 24 h and 81% ± 5%, N=6 at 48 h). ALP, and osteonectin levels were stable and collagen type I and OPN decreased over time, promoting osteogenesis. Despite the successful results with polymeric scaffolds, they also present several limitations, such as worse mechanical properties compared to metals (although elastic modulus of polymer scaffolds is closer to the bone values rather than the metal ones), and faster degradation, as well as the release of acidic compounds during the scaffold degradation that can cause adverse effects in cells over time. These drawbacks are related to pure polymeric scaffolds and consequently, the combination of polymers with other materials is under research as an alternative approach.

Most of the scaffold types described above can be applied to develop human osteoblast culture as they mimic bone topography and mechanical properties. Therefore, scaffolds are a good tool for bone regeneration and bone tissue engineering [93], and it would be expected that the most suitable materials for this purpose would be those naturally present in bone, like hydroxyapatite. However, as reported above, further studies need to be performed to improve some properties of these scaffolds. Besides, it is not unified which type of scaffold and material possesses the greatest characteristics for bone tissue engineering, which highlights the fact that more research is required to find a combined strategy with enhanced mechanical properties and cell biocompatibility with reduced drawbacks [75, 93].

3.2.3 Cell sheets culture

Cell sheets are an alternative approach for the use of scaffolds in organ and tissue engineering (Fig 8), which have gained a great interest over the last years. One of the advantages of this cell culture method is its capacity of making tissue transplants

(containing a high density of cells) without inflammatory reactions and with better recovery in comparison with other techniques [34, 94, 95]. Moreover, cell sheet culture avoids that a low survival rate occurs at the centre of the scaffolds and also that cells grow within endogenous ECM in the same sheet (cell-cell and cell-matrix interactions) [94-96].

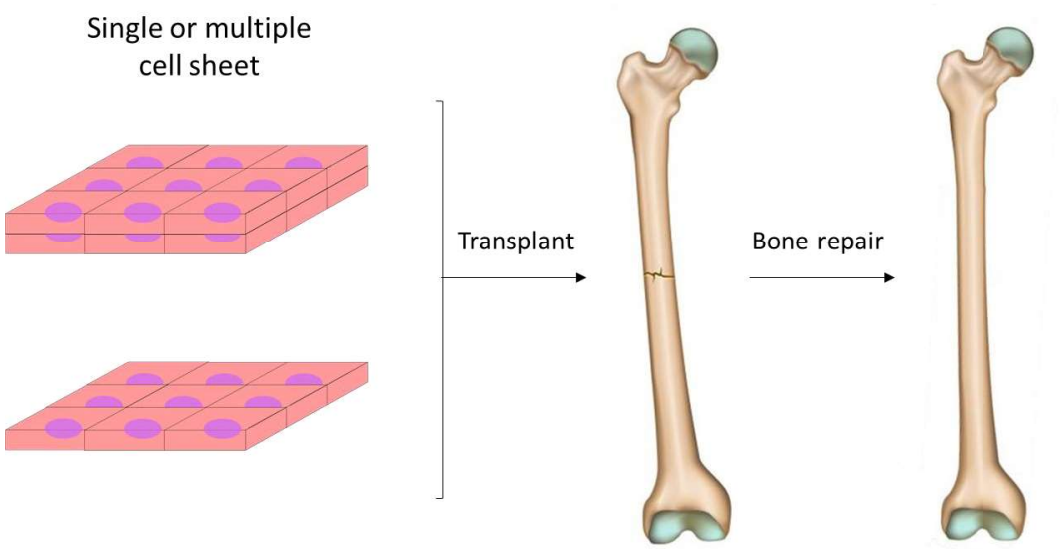


Figure 8. Schematic representation of the application of cell sheets in bone formation/regeneration.

The high number of studies which apply this *in vitro* model with cells from animals, like rats [97-103], rabbits [95], or pigs [104] demonstrates that this culture technique is getting more and more acceptance amongst researchers. In all of them, bone marrow stem cells were cultured as cell sheets that were transplanted followed by the analyses of bone formation/regeneration ability [97-103].

Some studies have applied this technique for culturing human bone cells, but, in any of the studies, osteoblasts have been employed. Cell sheets were generated with human bone marrow-derived mesenchymal stem cells, endothelial cells, and perivascular-like cells, to create a scaffold-free construct and study *in vivo* vessel formation, maturation, and stability [94]. Endothelial cells were bioprinted on cell sheets of osteogenically-differentiated human adipose-derived stromal/stem cells to create a prevascularised cell-based osseous construct [96]. Periodontal-ligament-derived cell sheets were cultured to study their safety and efficacy for future applications in the regeneration of periodontal tissues [105].

Cell sheets culture is becoming an interesting tool with good perspectives for *in vivo* applications. However, more studies are required to understand human bone formation and regeneration with different bone cell types, as currently, there are just a handful of research articles in this field.

3.2.4 Hydrogels

Despite hydrogels can be used as scaffolds, they are also considered as an alternative technique due to their particular properties [34, 106]. They are constituted by natural polymers, such as collagen, gelatine, fibrin, alginate, agarose, chitosan, or synthetic polymers such as poly (propylene fumarate-co-ethylene glycol), polyethylene glycol (PEG), polyethylene oxide and hydroxypropyl methylcellulose (HPMC) [106]. These 3D culture models, made of hydrophilic polymer networks, possess the ability to absorb a high amount of water similar to *in vivo* tissues. Moreover, they present good capability for cell encapsulation, adjustable biochemical, and mechanical properties, and cell biocompatibility with low risk of immune responses [34, 107]. Varying the composition of the hydrogels makes it possible to control and improve nutrient transport. However, there are not many studies that focus on gases diffusion, but oxygen diffusion seems to be a limiting factor for hydrogels cultures [108]. Different techniques of seeding cells on hydrogels to ensure high cell viability have been developed such as 3D printing, dropwise approach, direct mixing of cells with the hydrogel, and seeding cells on the hydrogel's surface (Fig. 9).

Several osteogenic cell lines (MG-63, SAOS2, and CAL72), as well as human primary osteoblasts, were cultured in a new hydrogel consisting of silated hydroxypropylmethylcellulose (Si-HPMC). Each cell type was suspended and mixed with the hydrogel in twelve well plates (Fig. 9). Cells grew as spheroids, without the presence of central necrosis, showing good viability and proliferation. Furthermore, osteoblasts presented a more differentiated state than in monolayer cultures [106].

A biodegradable sodium alginate hydrogel was stabilized with gelatine and overlayered with agarose and calcium salt of polyphosphate (polyP.Ca^{2+}- complex) which was developed for encapsulating 3D bioprinted SAOS2 cells (Fig. 9a). The young modulus of the scaffold was 13-22 kPa, which is very different from those reported for human bone. Cell proliferation was greatly increased due to the overlayer and the hydrogel components increasing the optical densities values from 0.49 ± 0.09 (time 0), to 1.42 ± 0.19 (3 days) and 2.98 ± 0.41 (6 days)), that induced a significant increase in osteoblast mineralization. This hydrogel is an interesting tool for future implant development and *in vivo* testing [109].

MG-63 cells were cultured in a biodegradable silk fibroin hydrogel (seeded by the dropwise approach (Fig. 9b)), containing hydroxyapatite crystals, to improve the mechanical and biocompatibility properties of the construct. The addition of the crystals to the hydrogel did not harm cell viability, proliferation, or differentiation; on the contrary, these three were enhanced. MG-63 cells grew as aggregates exhibiting a homogeneous distribution. The cytocompatibility of the hydrogel increased with higher mineral contents being a promising material for bone repair [110].

Biodegradable self-supporting hydrogels were generated with an ionic-complementary octapeptide (FEFEFKFK) containing 2, 3, 4, or 5% of water and an elastic modulus ≥10KPa. hOBs were mixed with the gel (Fig. 9c) resulting in a homogeneous suspension, being the hydrogel containing 3% water, the one with the best performance. This 3D model showed promising results due to the enhanced mineralization and good osteoblast viability which were able to proliferate and to develop an osteogenic state [64].

The mechanical and biological properties (compression modulus of 500 kPa) of poly (lactic-ethylene oxide fumarate) (PLEOF) hydrogels, cross-linked with poly (ethylene glycol)-diacrylate (PEG-Da) were improved by adding gelatine, that enhanced cell growth, and adhesion. hOBs were seeded on the gel surface (Fig. 9d). The increased porosity of the gels allowed hOBs to proliferate uniformly [111].

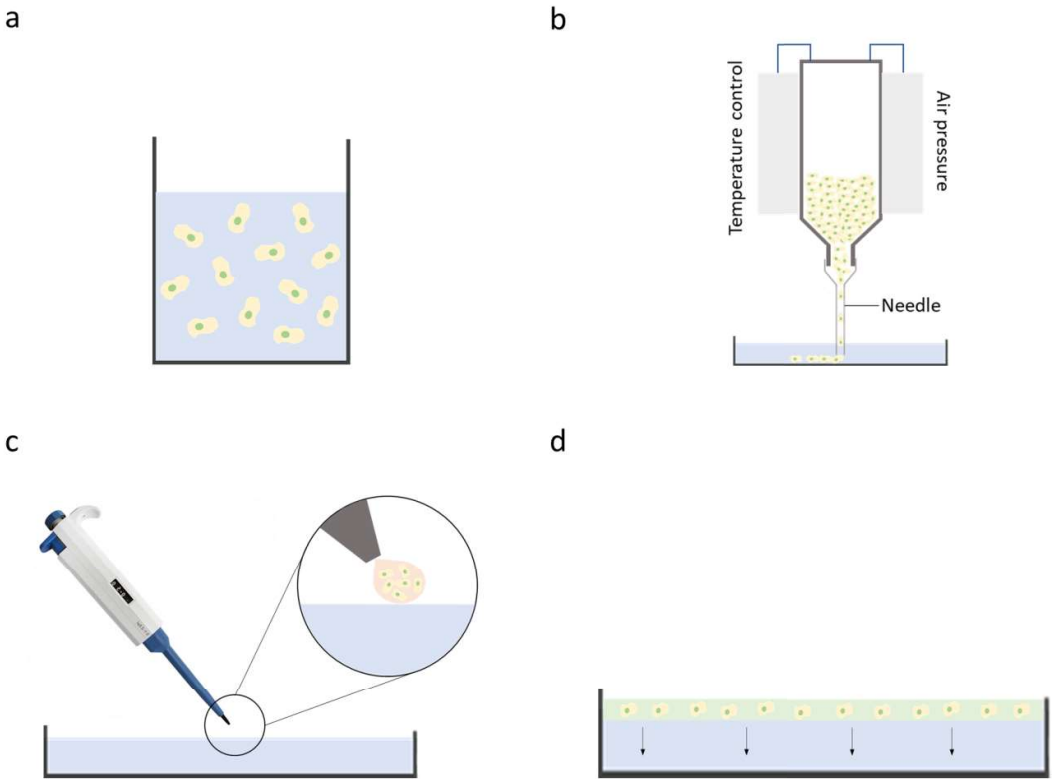


Figure 9. Schematic representation of different techniques of seeding cells on hydrogels: mixing cells with the hydrogel (a), 3D printing (b), dropwise approach (c), and seeding cells on the surface of the hydrogel (d).

There is a wide variety of hydrogels that have shown promising results in human osteoblast cultures. Similar to the scaffolds, the high number of studies testing different materials show that hydrogel bone cultures are in an early phase of research. Besides, most hydrogels still have limitations in cell proliferation and matrix production, their progressive degradation can modify their biochemical and mechanical properties like the stiffness is not similar to the human bone [34]. These factors make hydrogels less suitable for clinical application in bone regeneration. In some of the studies above mentioned,

different materials, like polyP.Ca^{2+}- complex or hydroxyapatite crystals, are added to pre-existing hydrogels, in an attempt to overcome these limitations. Cell source and culture conditions employed for osteoblast hydrogel culture are summarised in Table 7.

Table 7. Culture conditions applied in osteoblast primary cultures and cell lines when cultured on hydrogels. Key: αMEM, minimum essential media, DMEM, Dulbecco's modified eagle's medium, OGM, osteoblast growth media, FCS, foetal calf serum, FBS, foetal bovine serum, β-GP, β-glycerophosphate, Si-HPMC, silated hydroxypropylmethylcellulose, PLEOF, poly (lactic-ethylene oxide fumarate).

Cell type	Culture media	Supplements	Cell seeding density	Culture surface	Hydrogel stiffness	Aim of the study	Findings	Ref
Primary culture MG-63	αMEM with 5% FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (2D culture)	None	Unspecified	Si-HPMC-based hydrogel	Unspecified	Evaluation of a newly developed hydrogel for the 3D culture of osteogenic cells	Si-HPMC hydrogel was suitable for supporting osteoblastic survival, proliferation, and differentiation	[206]
CAL72 SAOS2	Hydrogel with 5% FCS. 1.5 ml of medium was overlaid onto the hydrogel containing cells	50 mg/ml of ascorbic acid, 10 mmol/L β-GP and 10^{-9}M dexamethasone	2 x 10^5 cells/1.5 ml					
SAOS2	McCoy's medium with 2 mM L-glutamine, gentamycin (50 µg/ml), with 5% FCS (2D culture)	10 nM dexamethasone, 50 mM ascorbic acid, and 5 mM sodium β-GP	10^5 cells/ml	25 cm^2 flasks	Unspecified	Study the effect of overlay agarose and polyP.Ca2+-complex onto a sodium alginate hydrogel on SAOS2 proliferation	Cell proliferation was greatly increased due to the overlayer and the hydrogel components that induced a significant increase in osteoblast mineralization	[209]
	McCoys's medium with FCS (hydrogel culture)		5 x 10^5 cells/ml	Sodium alginate hydrogel	13-14 KPa			
MG-63	DMEM with 10% FCS and 1% penicillin/streptomycin	None	10^5 cells/hydrogel	Silk fibroin hydrogel	Unspecified	Design of a silk fibroin hydrogel with hydroxyapatite crystals for bone tissue engineering	The hydrogel supported human osteoblat viability and proliferation, as well as improving cytocompatibility with increased mineral content.	[210]
hOB	DMEM with 10% FBS, 1% penicillin and 1% streptomycin	50 µg/mL ascorbic acid	1.5 x 10^5 cells/250 µl of hydrogel	Ionic-complementary peptide hydrogel	5.4 to 22.6 ± 1.2 KPa	Investigation of human osteoblast viability, proliferation, and mineralization in an ionic-complementary peptide hydrogel	Ionic-complementary octapeptides offered a suitable three-dimensional environment for osteoblastic cell function.	[64]

hOB	Complete OGM	None	2 x 10^5 cells/ml	PLEOF hydrogel	500 KPa	Study the incorporation of a naturally derived polymer (gelatine) into PLEOF hydrogels	Primary human osteoblast cells were adhered and proliferated in PLEOF-gelatin hydrogel	[111]

3.2.5 Small and large scale bioreactors

In vivo, bone cells respond to mechanical stimuli, and these signals have an important effect on bone remodelling. Static cell cultures fail in implementing these variables, as well as in providing a uniform nutrient and gas supply or metabolic waste removal, which can translate into negative effects on cells, like necrosis in the core of the scaffolds. Consequently, this is translated into a major obstacle in bone tissue engineering [112, 113]. For this reason, the use of bioreactors has been implemented in this field.

Bioreactors can be classified in four main types: i) rotating wall vessels, which is a horizontal culture system with rotating concentric cylinders and oxygenation through a coaxial tubular membrane (Fig. 10a); ii) spinner flasks, in which the medium flow is generated by a vertical stirrer or a magnetic stir where oxygenation occurs through the top of the vessel (Fig. 10b); iii) perfusion bioreactor, composed by perfusion chambers, containers or cartridges for cells/scaffolds, medium reservoir, a pump and a tubing system for oxygenation (Fig. 10c); iv) compression systems, which is consisting of a mechanical stimulation produced by one or more pistons (Fig. 10d). Besides, combined bioreactors from the above-mentioned are also under research [112, 113].

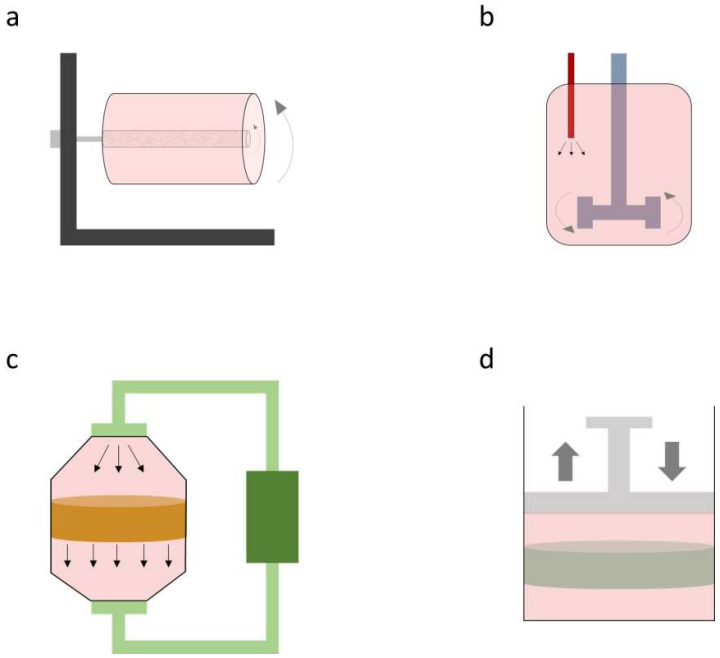


Figure 10. Schematic representation of the different types of bioreactors: rotating wall vessel (a), spinner flask (b), perfusion bioreactor (c), compression system (d).

A titanium alloy (Ti-6Al-4V) has been used to create different scaffolds (porosity of 51-76% and pore size of 400-1000 μm), where hOBs were seeded and cultured in both dynamic and static conditions. The dynamic conditions consisted in placing the scaffolds in specific retainers inside perfusion chambers, with a flow rate of 50 μL/min. Moreover,

there were gas permeable silicon tubes for CO_2 exchange. Dynamic cell culture conditions showed an improvement in cell migration through the porous titanium scaffold, compared to static conditions. However, they could not determine which conditions were more suitable for increasing proliferation [87].

Monocultures of MG-63, U-2 OS, SAOS2, hFOB cells, and rat calvaria primary osteoblasts were grown in a rotating wall vessel bioreactor. In this work, the effect of microgravity, employing Clinostat, a device that neutralized the effects of gravitational pull thanks to rotation forces, was investigated. Microgravity led to the inhibition of proliferation, affecting the cell cycle by altering the structure of spindle microtubules and gene expression [114].

In a perfusion bioreactor consisting of a flask, silicone tubing, and peristaltic pump, MG-63 cells were seeded in ceramic scaffolds with a pore diameter ranging from 500–630 μm. Perfusion flow was set at 3 ml/min in two different directions, convergent and divergent flows. Both flows increased cell survival, and proliferation was improved in comparison with static cultures. Nevertheless, cultures with a convergent flow showed a better performance than those exposed under divergent flow [115].

Human foetal chondrocytes and hFOBs were seeded in PGA scaffolds and co-cultured in recirculation column bioreactors in which was combined a perfusion system (made of a silicone tubing and a pump) with a rotating system based on a magnetic stirrer. This coculture exhibited higher collagen concentrations than the cartilage cultures used as control. The chondrocytes layer presented glycosaminoglycan production and the osteoblast layer showed mineralization. Cartilage generation occurred when coculturing chondrocytes in contact with osteoblast [116].

In conclusion, bioreactors can be used to generate dynamic cell cultures with enhanced osteoblastic parameters, such as proliferation, viability, gene expression, and mineralization. Based on this, bioreactors are a promising tool for improving the performance of scaffolds and overcoming some of the current limitations in bone tissue engineering. Cell types, culture medium, and supplements employed for osteoblast culture in bioreactors are summarised in Table 8.

Table 8. Culture conditions utilised in osteoblast primary cultures and other cell lines using bioreactors. Key: MEM, minimum essential media, DMEM, Dulbecco's modified eagle's medium, RPMI-1640, Roswell Park Memorial Institute 1640 medium, M199, Gibco media 199, Ham's F12, Gibco Ham's F-12 Nutrient Mixture, FBS, foetal bovine serum, FCS, fetal calf serum HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, βGP, β-glycerophosphate, G418, Geneticin, PGA, polyglycolic acid, PLGA, poly(lactic-co-glycolic) acid.

Cell type	Culture media	Supplements	Cell seeding density	Culture surface	Aim of the study	Findings	Ref
hOBs	MEM with 10% FCS, 1% penicillin/streptomycin, 1% amphotericin and 1% HEPES buffer	100 mM dexamethasone, 50 mg/ml L-ascorbic acid and 10 mM βGP	4 x10^5 cells in 100 µl per scaffold	Ti-6Al-4V	Investigation of the influence of different scaffold configurations and cultivation methods on hOBs	All scaffolds showed an increase in cell activity and matrix production under static conditions, and dynamic culture enhanced cell migration	[87]
MG-63	DMEM) with 10% FBS, high glucose (4.5 g/l), penicillin (100 U/ml), streptomycin (100 µg/ml) and 1% of 200 mM L-glutamine	None	10^7 cells/scaffold	Beta tricalcium phosphate cylinders	Evaluation of the efficiency of a new bioreactor for the *in vitro* development of large bone substitutes	Convergent and divergent flows increased cell survival and proliferation, but cultures with a convergent flow showed better performance than those exposed under divergent flow	[115]
SAOS2	McCoy's 5A modified medium with, 15% FBS, L-glutamine and HEPES, 2% sodium pyruvate and 1% antibiotics	10^{-8} M dexamethasone and 10 mM βGP	7 x 10^5 cells/scaffold	Porous polyurethane scaffolds	Analyses of the effect of a polyurethane porous scaffold and perfusion bioreactor in SAOS2 proliferation and mineralization	Polyurethane scaffolds with perfusion enhanced osteoblast proliferation and mineralization	[117]

hFOB	DMEM with FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 0.125 µg/ml amphotericin B	50 mg/l ascorbic acid	2.0 - 2.8 10^6 cells/flask (75cm^2) and 2.2 x 10^7 cells/scaffold	25 cm^2 and 75 cm^2 flasks, and PGA scaffolds	Examination of the culture of chondrocytes and osteoblasts into PGA scaffolds and recirculation column bioreactors to produce tissue-engineered cartilage	Composite osteochondral constructs were successfully engineered in recirculation column bioreactors, exhibiting beneficial effects on generating cartilage tissues in contact with bone cells.	[116]

3.2.6 Microfluidics

The development of micro- and nanoscale fabrication has allowed the creation of a new type of cell culture system, close to the bioreactors but on a smaller scale, known as microfluidics. Microfluidic chips allow us to engineer microscale complex structures with well-controlled and defined parameters, like dynamic microenvironments, that mimic closely the *in vivo* conditions (Fig 11). Microfluidic chips are also known as lab-on-a-chip or organ-on-chip, as they consist of a small device with intricated structures and chambers that replicates the *in vivo* conditions and functions of a specific organ or tissue [34, 118, 119].

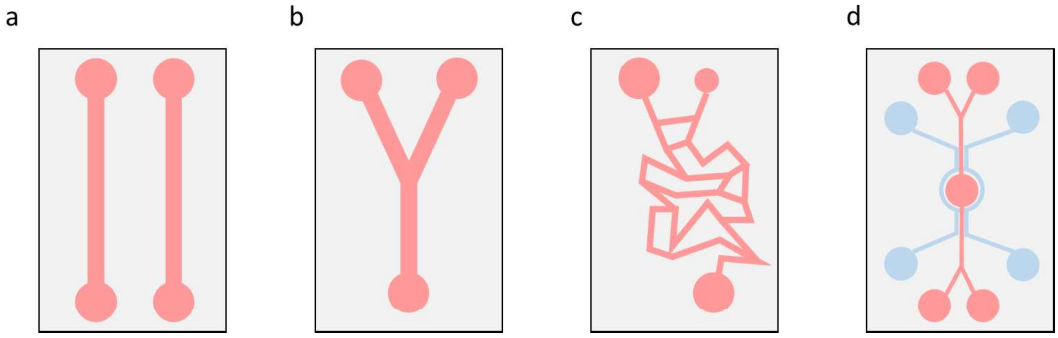


Figure 11. Examples of microfluidic cell culture systems or chips: linear channels (a), bifurcating channels (b), microvascular networks (c), and idealized networks (d).

The growth of cell cultures on these devices is controllable, reproducible, and can be optimized. Moreover, microfluidic chips benefit from: i) a reduced cost, as small volumes are employed; ii) a high capacity of implementing flow or perfusion (Fig 12); iii) integration of multiple processes within the same device, like cell culture growth, cell sampling, fluid control, cell capture, cell lysis, mixing, and detection, and iv) a high capacity of developing spatially controlled cocultures [118, 119].

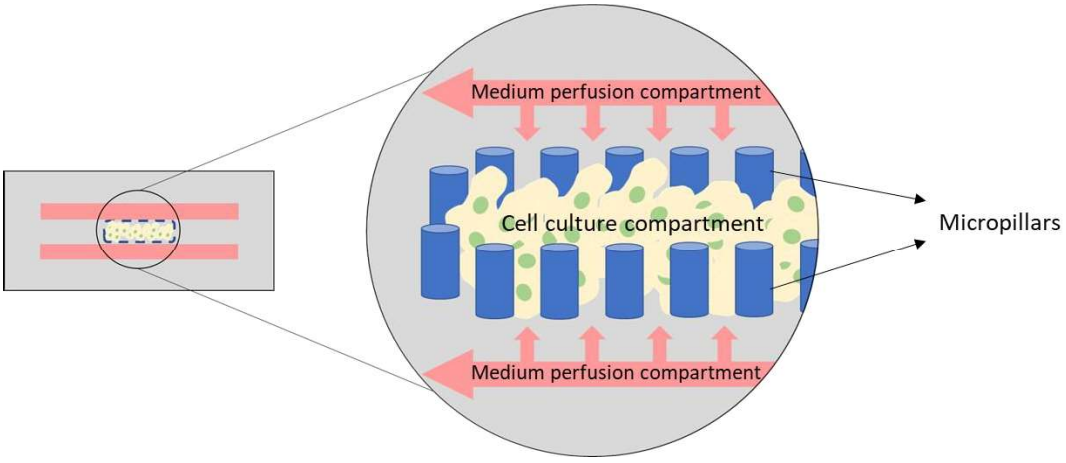


Figure 12. Microfluidic perfusion 3D cell culture system. Cells are separated from the medium by micropillars, through which the medium perfuse, but cells cannot go through and are retained within the cell compartment of the microfluidic chip.

Methacrylated gelatin (GelMA) and alginate were combined for the fabrication of a microfluidic-based fiber system. It consisted of double-layer hollow microfibers, where HUVECs were encapsulated in the middle layer, replicating a vascular vessel, and MG-63 cells were located in the outer layer, in a bone-like environment. Different flow rates, 5, 10, 20, 30, and 40 µl/min, were applied. Osteoblasts showed good viability, vigorous growth, and increased gene expression (higher levels of collagen type I and bone morphogenetic protein (BMP-2)) [120].

The isolation of an acceptable amount of specific bone cells from *in vivo* samples remains challenging. A microfluidic system with an electric field cage (based on negative dielectrophoresis) was employed for the isolation of human osteoblasts, which were trapped when they went across a planar ring electrode, while the other cell type were repelled. MG-63 cells were isolated and recovered, presenting a 100% purity using this system [121].

Rebl et al. analysed the MG-63 behaviour and metabolism in a new sensor chip modified with plasma polymerized allylamine (PPAAm), a chemically treated surface, incorporated due to its positive effects on cell growth. MG-63 cells showed an enhanced adhesion on the device, and similar acidification and oxygen consumption compared to control chip surfaces (without PPAAm), inferring that the plasma treatment in the sensor chip surfaces results in an enhanced cell adhesion without altering their metabolism [122].

The viability of SAOS2 cells on polydimethylsiloxane Sylgard 184 microfluidic chips, with microchannels of 200 µm, was evaluated. Particles of different materials (polystyrene, PDMS, PDMS with carbon nanotubes, rough PDMS, silanized PDMS, Cyclo-Olefin (COC), and epoxy resins) were added to evaluate their biocompatibility. Cell proliferation and viability were higher with COC and rough PDMS, being these materials the most biocompatible for SaOS2 cells [123].

The central chamber of microdevices of PDMS, consisting of two media channels located on either side of a central channel, were inoculated with a collagen-based hydrogel and hOBs. A chemical gradient of platelet-derived growth factor β (PDGF-BB, a chemotactic factor for osteoblasts migration) was also added. The PDGF-BB gradient stimulated hOBs migration velocity at lower doses, while at higher doses directionality was faintly increased. Besides, the addition of transglutaminase to the hydrogels enhanced directional cell migration without altering their motility [124].

The use of microfluidic devices allows the development of more complex microenvironments with human osteoblasts compared to other conventional culture techniques and provides the possibility of mimicking more reliably the real *in vivo* conditions with great ease to control mechanical (surfaces) and biochemical parameters

(gradient of growth factors). A bone-on-chip culture is a revolutionizing platform amongst the 3D culture techniques; however, it is necessary to pave the way to make them more biocompatible and easy to use and implement them in testing laboratories. From a future perspective, the development of a reliable bone-on-chip is crucial to be part of the recreation of a whole *in vitro* human body with a high level of complexity [36]. Cell types, culture medium, and supplements employed for osteoblast culture in microfluidic systems are summarised in Table 9.

Table 9. Cell culture medium and supplements utilized in osteoblast primary cultures and other cell lines with microfluidics. Key: DMEM, Dulbecco's modified eagle's medium, OGM, osteoblast growth medium, FBS, foetal bovine serum, FCS, foetal calf serum, COC, Cyclo-Olefin, PDMS, polystyrene, PDGF-BB, platelet-derived growth factor β.

Cell type	Culture media	Supplements	Cell seeding density	Culture surface	Aim of the study	Findings	Ref
MG-63	DMEM with 1 % penicillin/streptomycin, and 10 % FBS	None	5 x 10^6 cells/ml (bioreactor)	Dishes and methacrylated gelatin and alginate microfibers	Generation of functional microfibers (methacrylated gelatin and alginate) to obtain biomimetic complex bone tissue engineering	Osteoblasts cultured in a microfluidic-based fiber system showed high viability, vigorous growth, and increased gene expression	[120]
MG-63	DMEM with 10% FCS	None	10^6 cell/ml (microfluidic system)	Suspension (microfluidic channels) and 384-well plates (recovering)	Study of a new system for the isolation, concentration, separation, and recovery of human osteoblast-like cells from a heterogeneous population	The developed microfluidic system allowed the isolation and recovery of osteoblasts with a 100% purity	[121]
MG-63	DMEM with 10% FCS, and 1% gentamicin	None	6 x 10^4 cells/chip	Sensor chips (Bionas® SC 1000) modified with plasma polymerized allylamine	Analysis of the metabolic activity of osteoblasts and their time-dependent adhesion profile	The method developed was suitable for the analyses of the osteoblast behavior after plasma-chemical surface treatment.	[122]
SAOS2	DMEM with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 4 mM L-glutamine	None	6 x 10^3 cells/well	75 cm^2 flasks and seven different supports placed in 96-wells plates	Design and fabrication of microfluidic devices for pharmacological *in vitro* drug testing	Cell proliferation and viability were higher with COC and rough PDMS, being the most biocompatible materials for SaOS2 cells	[123]
hOB	OGM with 10% of FCS	None	1 x 10^5 cells/ml	Collagen hydrogel	Evaluation of the impact of extracellular matrix properties and growth factor gradients on 3D osteoblast motility and the role of cell-matrix degradation	The PDGF-BB gradient and the addition of transglutaminase to the hydrogels had a stimulating effect on hOBs migration	[124]

4. Conclusions and future perspectives

Since the first human osteoblast cultures in the 70s, a wide range of 2D cell culture models has been developed moving forward towards more advanced 3D culture models in the last decades. 3D culture models have succeeded in mimicking more closely the bone microenvironment which facilitates the clinical translation of novel medicines to treat bone diseases; however, no cell culture model fits all *in vitro* testing due to the diverse osteogenic functionality which limits the comparison of results amongst the broad scientific community. Many different factors such as cell line, cell culture media, substrate micro-patterning, and stiffness are affecting significantly the outcome.

Advances in technology have allowed the development of more complex structures such as 3D printing, bridging the gap between *in vitro* and *in vivo* models for bone tissue engineering. Nevertheless, one of the biggest challenges in bioprinting still is to recreate the hierarchical complexity of the bone, including the appropriate mechanical and biochemical stimulus for guided cellular differentiation. Besides, the development of novel materials more biocompatible, biodegradable, and with optimal mechanical properties is fundamental when constructing 3D scaffolds. The price of bioprinters is getting lower in the last years becoming affordable for a greater number of researchers. This widens the landscape of 3D bone culture models which in combination with microfluidic chips seem to be the way forward to mimic the bone microenvironment with higher accuracy and reproducibility.

Acknowledgments

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