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DELIVERY OF PHARMACEUTICS TO BONE: NANOTECHNOLOGIES, HIGH-THROUGHPUT PROCESSING AND *IN SILICO* MATHEMATICAL MODELS

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Abstract

In the last decade, nanobiotechnology research has emerged as a revolutionising new approach to the 21st century pharmaceutical challenges, offering valuable gains in a vast set of biomedical applications. In the field of bone tissue engineering, a broad range of nanotechnology-based delivery systems have been researched and the most recent developments in high-throughput technology and *in silico* approaches are creating very high expectations.

This review presents a comprehensive overview of the emergent nanotechnology-based materials, processing techniques and research strategies for the delivery of pharmaceutics to bone including the materials general characteristics and the available drug delivery systems to distribute agents systemically or locally. Complementary to what was stated above, it also reviews the latest high-throughput processing techniques and the existent *in silico* tools (mathematical and computational models) used to help on the design of delivery systems.

Keywords: Bone tissue engineering, targeted drug delivery strategies, biomaterials, high-throughput processing technology, *in silico* tools, nanotechnology.

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Introduction

A full list of abbreviations used is given in Table 1. Presently, medics dealing with skeletal diseases have a wide variety of therapeutic agents at their disposal. Factors such as, antibiotics, anticancer, anti-inflammatory, growth factors, enzymes, antibodies, bioactive proteins, cells and non-viral genes (DNAs and RNAs) are currently being addressed for the treatment of bone diseases (Bose and Tarafder, 2012). Still, most of the currently available clinical therapies have significant drawbacks associated and there is no effective treatment for some of the most common bone disorders such as osteoporosis, osteoarthritis, osteomyelitis, infections, bone cancer and fracture repair (Gu *et al.*, 2013). In most cases, the balance between medication side-effects and treatment efficacy remains a major issue and there is a high demand for delivery vehicles that can provide adequate, sustained and localised presentation of drugs in a time-dependent manner.

The majority of the current drugs are administered orally or parenterally but when administered alone, drugs are usually rapidly cleared from circulation showing very low bioavailability and strong limitations in the efficient delivery to the required site of injury (Gittens *et al.*, 2005; Hirabayashi and Fujisaki, 2003; Takahashi-Nishioka *et al.*, 2008; Uludag, 2002; Zhang *et al.*, 2007). In order to overcome this, large doses must be taken with the consequent increase of the toxicity risk. As an example, most of the current anti-osteoporotic drugs are administered at higher doses to account for pharmacological interactions and expose the patients to adverse effects such as the endometritis, cancer risks and intrauterine haemorrhage on women who underwent prolonged oestrogen therapy (Rossouw *et al.*, 2002). Parathyroid hormone (PTH), a Food and Drug Administration (FDA) approved drug, is recommended to be administered only to severe cases for a maximum period of two years (Tashjian and Goltzman, 2008; Watanabe *et al.*, 2012). Bisphosphonates (BPs) oral therapy has been related with gastritis, gastric ulcer and erosive esophagitis (Graham, 2002; Naniwa *et al.*, 2008). Moreover, as anti-osteoporotic drugs may have to be taken for prolonged times, there is also a problem with patient therapy compliance. Other types of administration such as intravenous, nasal and transdermal delivery have also been addressed. Several drugs such as BPs, PTH and calcitonin can be administered intravenously to avoid first pass effects (Dodson, 2009; Schipper *et al.*, 1994; Shi *et al.*, 2015; Stirling *et al.*, 1991; Tam *et al.*, 1982; Tsavaris *et al.*, 2006) but, because these drugs need to be given on a daily basis, this method of administration is inconvenient

Table 1. List of abbreviations used in text and tables.

3D	three-dimensional
5TGM1	mouse myeloma cell line
Alen	alendronate
alpha-AL-HPLN	ALCAM-hybrid polymerised liposomal nanoparticle immunoconjugate
ASCs	adipose-derived stem/stromal progenitor cells
AspSerSer(6)	six repetitive sequences of aspartate-serine-serine
ATO	arsenic trioxide As_2O_3
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BPs	bisphosphonates
B-TCP	beta tricalcium phosphate
CaP	calcium phosphate
CaPC	calcium phosphate cement
CaS	calcium Sulphate $CaSO_4$
CFS	antibiotic ceftriaxone-sulbactam sodium
CH	chitosan
CH-TPP	chitosan-tripolyphosphate
CS	chondroitin sulphate
DMOG	dimethyloxaloylglycine
DOTAP	dioleoyl trimethylammonium propane
Dox	doxycycline
DSPC	distearoylphosphatidylcholine
ERK1/2	extracellular signal-regulated kinase
EWS	Ewing sarcoma
FDA	Food and drug administration
FGF	fibroblast growth factor
Fli-1	Friend leukemia integration 1 transcription factor
FTY720	selective agonist for sphingosine 1-phosphate receptors
HA	hyaluronic acid
Hap	hydroxyapatite
HEK293	human embryonic kidney cells
hMSCs	human mesenchymal stem cells
IGF-1	insulin-like growth factor 1
iPSCs	pluripotent stem cells
LDH	layered double hydroxide
Lov	lovastatin
MC3T3-E1	pre-osteoblastic cell line
MCF-7	human breast cancer cell line
MBG	mesoporous bioactive glass
MG63	human osteosarcoma
MNU	N-methyl-N-nitrosourea
mPEG	polyethylene glycol monomethyl ether
MS	mesoporous silica
MTX	methotrexate
ND	nanodiamonds
NFKβ	nuclear factor kappa B
NO	nitric oxide
NP	nanoparticle
NS	nanospheres
OPG	osteogenic growth peptide
OVX	ovariectomised
PAH	poly (allylaminehydrochloride)
PC3	human adenocarcinoma of the prostate
PEG	polyethylene glycol
PCL	polycaprolactone
PCLA	poly (ε-caprolactone-co-lactide)
PEG	polyethylene glycol
PEI	polyethylenimine
PLA	polylactic acid
PLA-DX-PEG	poly-d l-lactic acid-p-dioxanone-polyethylene glycol block co-polymer
Plekho1	casein kinase-2 interacting protein-1
PLGA	poly(L-lactide-co-glycolide)
PLK 1	polo-like kinase 1
PLL	poly-L-lysine
PLLA	poly-L-lactide
PTH	parathyroid hormone
PTX	paclitaxel
Pur	purmorphamine
RA	rheumatoid arthritis
RAW 264	mouse macrophage cancer cell line
RANK	receptor activator of nuclear factor κ B
RES	reticuloendothelial system
rhBMP-2	Recombinant human bone morphogenetic protein-2
Ris	risondronate
RNA	ribonucleic acid
RNAi	RNA interference
Ros	rosuvastatin
RPMI 8226	human myeloma cell line
Saos-2	human osteosarcoma cells
shRNA	short hairpin RNA
Sim	simvastatin
siRNA	small interfering RNA
SPIONs	superparamagnetic iron oxide nanoparticles
Src	proto-oncogene tyrosine-protein kinase
SMO	proto-oncogene tyrosine-protein kinase
T47D	breast cancer cell line
TCaP	tricalcium phosphate
TGF	transforming growth factor
TGF ss1	transforming growth factor ss 1
Ti	titanium
VCM	vancomycin
VEGF	vascular endothelial growth factor
Zol	zoledronate

and difficult to follow through. Calcitonin is also available as a nasal spray, but the small surface area available and the rapid mucociliary clearance is very limiting (Dodson, 2009; Schipper *et al.*, 1994; Stirling *et al.,* 1991; Tam *et al.,* 1982; Tsavaris *et al.,* 2006). Oestradiol can be delivered transdermally, by application to the skin, but not many drugs possess the physicochemical properties for this type of delivery (Shuid *et al.*, 2013).

Currently, other than seeking new agents, efforts should be made in the development of innovative approaches that enable a specific and targeted delivery of therapeutics to bone and, therefore, improve drug efficiency and patients compliance while minimising adverse side-effects.

Despite having a fairly simple microscopic structure composed of 50-70 % mineral (primarily hydroxyapatite), 20-40 % organic matrix (mainly collagen type I), 5-10 % water and 1-5 % lipids, bone repair/regeneration entails significant challenges due to bone´s complex geometries and highly hierarchical and integrated structures, including hard and soft tissues (Ruppel *et al.*, 2008). Such structure, combined with the existence of a membrane of lining cells, which form a marrow-blood barrier and

prevent the access of large exogenous substances to the bone surface (Travlos, 2006), makes the development of bone-specific delivery systems a difficult task.

Controlled drug delivery systems became available in the early 70s and are now a dynamic field of research (Jeong *et al.*, 2002; Qiu and Park, 2001). The most recent efforts are looking into the development of multifunctional and stimuli-sensitive nanoparticles with well-defined structures/patterns that allow the combined delivery of different therapeutic agents (such as anti-bacterial agents and osteogenesis and angiogenesis stimulators) in response to specific local stimuli (such as temperature, pH, ionic strength, ions, *etc.),* thus potentiating the therapeutic results (Caldorera-Moore and Peppas, 2009; Tautzenberger *et al.*, 2012; Torchilin, 2014).

To be considered a drug carrier, the material must be non-toxic (*i.e.* bioinert or biodegradable), biocompatible, able to incorporate a drug either physically or chemically, retain the drug up to the specific target without causing side effects in other cells or tissues, and have the capacity to deliver the drug in a controlled time manner (Bose and Tarafder, 2012).

In the most recent strategies, different types of materials from biologics, polymers, silicon-based materials, carbon-based materials, metals, or combinations of them, structured in nanoscale formats are being considered (Satarkar and Zach Hilt, 2008).

The choice of material depends on the therapeutics chemical characteristics (*i.e,* hydrophobicity or hydrophilicity, molecular size, isoelectric point, *etc.),* on the delivery rate intended, on whether materials degradation is desired or not, on the targeted bone, on the targeted cell(s) and on the size of the injury. Complementary, also the carrier´s structure, molecular weight, and drug release mode (such as by passive diffusion, carrier degradation, or degradation of a linker that connects the drug and carrier) have major effects on the efficacy of the drug delivery in a context-specific manner. Higher molecular weight carriers present longer circulating half-lives (that putatively potentiate the drug accumulation at the targeted diseased sites) but also an increased uptake by the reticuloendothelial system (RES) (Pan *et al.*, 2008; Wang *et al.*, 2006). Therefore, the carrier´s molecular weight, and ultimately its hydrodynamic radius, is a very important factor to take into account in drug delivery systems. Whenever the delivered therapeutics may be degraded by lysosomal enzymes, and does not create biocompatibility issues following RES uptake, high molecular weight and high targeting moieties may be advantageous over smaller ones. On the contrary, in cases where the drug´s metabolism by the RES is a concern, lower molecular weight carriers with lower percentages of targeting moieties can be recommended (Low and Kopecek, 2012).

By presenting a similar size to bone ultrastructure, nanoparticles contain great potential as bone-specific carriers. The unique physicochemical properties of nanostructured biomaterials, such as being ultra-small and of controllable size, large surface area to mass ratio, presenting high reactivity and functionalisable structure has been widely explored by pharmaceuticals as means to circumvent some of the limitations of conventional drug delivery methods. In fact, when compared with traditional methods, drug delivery systems using nanoparticles present several advantages: i) circulation time is considerably increased due to the capacity to protect the drug from degradation and avoidance of its renal excretion; ii) drug bioavailability is improved and the solubilisation of lipophilic drugs is enhanced (due to the large surface area); iii) drug release can be performed in a controlled and sustained manner, with the consequent reduction on plasma level fluctuations and side-effects; and vi) drug delivery to specific sites, improving the protection of non-target tissues and cells *(*Moghimi *et al., 2001;* Papahadjopoulos *et al., 1991; Torchilin, 2005)*. Moreover, the surface modification of nanoparticles by polyethylene glycol (PEG) chains was shown to increase the circulation time (Klibanov *et al.*, 1990) and is highly explored for the passive delivery of drugs such as the anticancer ones (Gabizon *et al.*, 1994; Matsumura and Maeda, 1986). Still, taking into consideration the above described features of higher molecular weight carriers, depending on the application, macromolecular and small molecule drug delivery should also be considered (Low and Kopecek, 2012).

The drug loading in a carrier may be achieved by many means such as adsorption, incorporation, inclusion and connection through degradable linkers. The use of degradable linkers may be of upmost value for the tuning of the drug release profile. By inducing chemical modifications on the linker, either an immediate administration of high amounts of drug or small amounts of drug administered over an extended period of time may be obtained (Low and Kopecek, 2012). Moreover, by including responsive linkers for bone targeting such as specific enzymes cleavage sites or pH-sensitive sequences, a precise site-specific release of the drug may be achieved (Mullen *et al.*, 2014; Such *et al.*, 2015; Wang *et al.*, 2013). Several studies using sensitive linkers to metalloproteinases (expressed by osteoclasts, osteoblasts and overexpressed in many bone metastasis) (Hu *et al.*, 2011; Liu *et al.*, 2015; Takaishi *et al.*, 2008), cathepsin K (expressed at resorption lacunae) (Segal *et al.*, 2009), cathepsin B (Ogbomo *et al.*, 2013), and acid-sensitive linkers (such as hydrazone bonds) (Xu *et al.*, 2015) have been shown to be effective for the drug release at specific cells/sites. In addition, characteristics such as elongated spacers (Miller *et al.*, 2009; Miller *et al.*, 2008; Segal *et al.,* 2009) and disulphide linkers (Kowalczyk *et al.*, 2012; Kurtoglu *et al.*, 2009) have also been considered for drug release into bone.

Presently, the number and complexity of the different therapeutics/biomaterials/technology combinations being investigated for targeted drug delivery in bone is very extensive. Although the effects of many of these systems have been widely approved in animal models, so far only very few have been approved for clinical use (Anderson *et al.*, 2014). Still, considering the large number of *in vitro* and *in vivo* preclinical studies currently ongoing this is now a very promising field of research.

Following, we provide an overview of the currently available approaches to drug delivery to bone. The pipeline advancements in computational and high-throughput approaches will be discussed. Cell-based therapies will not be encompassed.

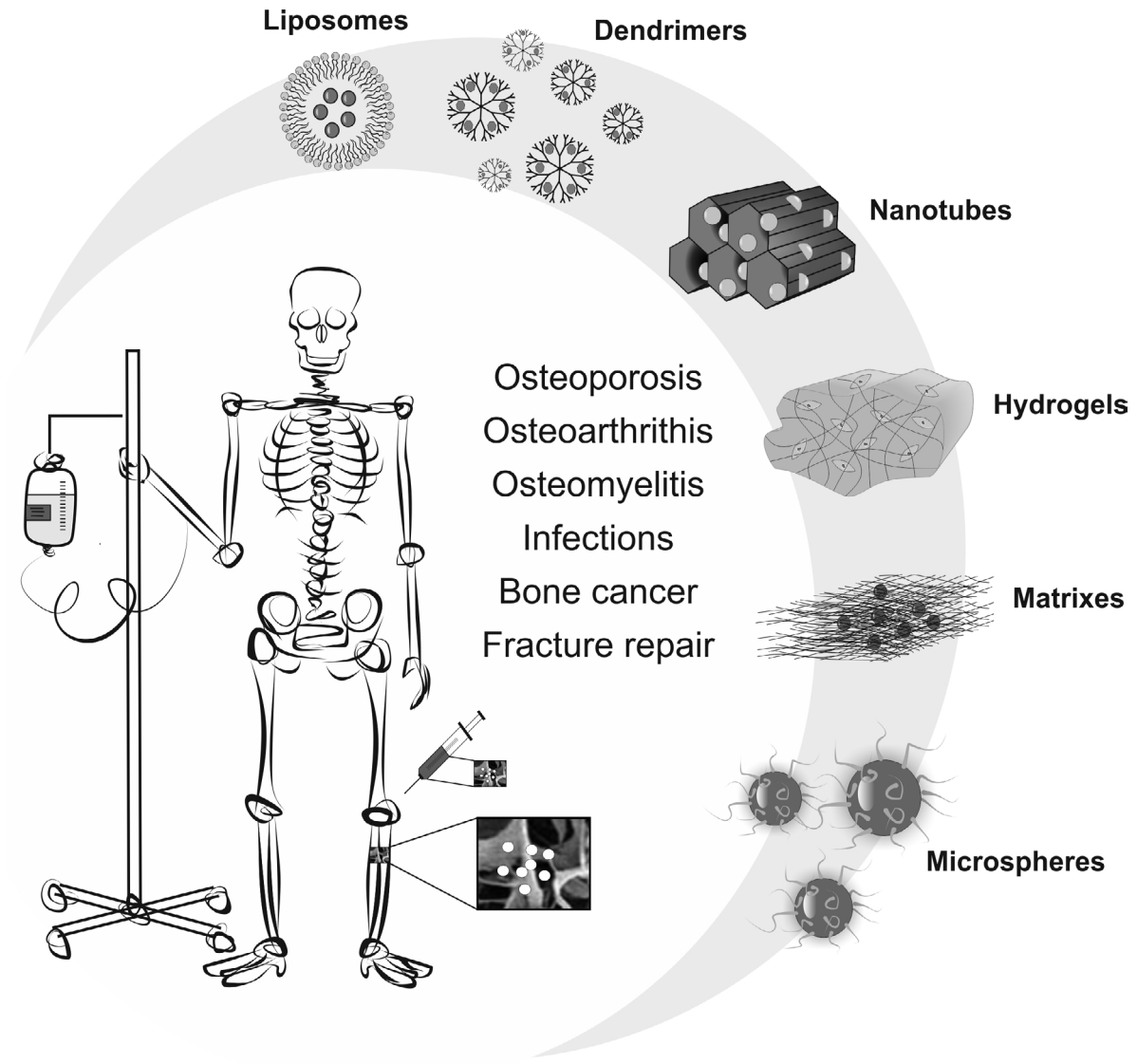

Fig. 1. Schematic overview of the options for targeted drug delivery to bone.

Drug delivery systems to bone

Systemic bone-related diseases (such as osteoporosis and osteoarthritis) may be better addressed with a systemic delivery system that releases the drug specifically to a broad range of bones in the body. Conversely, a localised injury (such as a critical fracture) may require a local delivery of the therapeutics that can be accomplished either by surgical implantation or by injection of the material at the targeted bone site (Fig. 1).

Systemic drug delivery systems

Considering the very wide diversity of patients' conditions, bone targeting delivery systems for systemic administration would represent a less invasive route of application, being therefore ideal and more convenient. Drug carriers for systemic drug administration usually enable i) prolonged circulation time in plasma, ii) distribution and accumulation at the targeted tissue and iii) protection of the drug from enzymatic and chemical degradation. Different physical parameters such as particle size (Alexis *et al.*, 2008; Decuzzi *et al.*, 2010; Petros and DeSimone, 2010), shape (Decuzzi *et al.,* 2010; Geng *et al.*, 2007; Tao *et al.*, 2011), rigidity and softness (Merkel *et al.*, 2011) have been shown to affect the particles' pharmacokinetics, targeting ability and cellular uptake. A significant number of researches have been focusing on active and passive targeting of systemically administrated particles to bone.

Oral administration

Successful oral delivery requires the protection of the drug-loaded particles from degradation in the gastrointestinal tract and their safe transportation across the intestinal membrane. The mechanisms of micro- and nanocarriers oral uptake have been extensively investigated. Studies showed that nano- and microparticles are easily taken up by a group of localised endothelial cells in the small intestine, especially by Peyers patch (des Rieux *et al.*, 2006; McClean *et al.*, 1998), with the consequent increase in the drug absorption. In addition, the incorporation of the drug into a particle core can protect it from the gastrointestinal

tract environment and thus increase the drug stability (Werle *et al.*, 2006) and extend the blood circulation time of the drug (Leane *et al.*, 2004).

Calcitonin and PTH are among the two most explored drugs in oral delivery systems for osteoporosis. Mucoadhesive devices (Gupta *et al.*, 2013; Kawashima *et al.*, 2000) and liposomal formulations (Garcia-Fuentes *et al.*, 2005), poly(L-lactide-co-glycolide) (PLGA) nanoparticles (Sang Yoo and Gwan Park, 2004) and pH-sensitive microspheres (Lamprecht *et al.*, 2004) have been tested *in vitro* and *in vivo* for the oral delivery of calcitonin, showing enhanced and sustained release of the drug. For the oral administration of PTH, PEGylated and thiolated chitosan nanoparticles loaded with PTH 1-34 showed increased drug half-life and improved bioavailability when compared to the bare peptide that is delivered systemically for treating osteoporosis (Narayanan *et al.*, 2014; Takeuchi *et al.*, 2003). In a different study, PTH 1-34 encapsulated in an oral microemulsion delivery system (85:15, oil/water) consisting of Labrasol, Crodamol GTCC, Solutol® HS 15, d-tocopheryl acetate (6:2:1:1, w/w) and saline water showed high drug loading efficiency (83 %) and permeability, and significantly higher resistance to proteolysis *in vitro*. The oral administration to osteoporotic rats showed increased bioavailability and improved bone microstructure (Guo *et al.*, 2011).

Also in the treatment of autoimmune diseases such as rheumatoid arthritis, the use of biodegradable microparticles – first promoted for vaccine development – is now being addressed as an attractive option for an oral administration of auto antigens and inducing of oral tolerance (des Rieux *et al.*, 2006). Kim *et al.* (2002) showed that a single administration of PLGA nanoparticles entrapping type Π collagen could induce oral tolerance more efficiently than repeated oral administrations of intact type Π collagen. From the same lab, Lee *et al.* reported that the conjugation of PEG with immunodominant peptides for collagen-induced arthritis showed promising results as a delivery method for the induction of oral tolerance, rather than the whole Type II collagen and peptide (Lee *et al.*, 2005).

Delivery via the vascular system

Depending on the final objective, either the mineral portion of the bone (mainly composed of hydroxyapatite) or the bone marrow portion (constituted namely by progenitor cells and lipoproteins) can be targeted.

Targeting bone´s mineral fraction

Systemic delivery of therapeutics to bone can be achieved mainly by enhanced permeability and retention phenomenon, a result of the increased vascular pressure caused by the cytokines present in inflamed tissues or extravasation through vessels in or near the bone (Fang *et al.*, 2011; Maeda, 2010; Maeda *et al.*, 2009; Torchilin, 2011). As bone´s vasculature is fenestrated with pore sizes up to 80 nm (Howlett *et al.*, 1984) (exceeding the hydrodynamic size of most circulating nanomedicines), the targeting of hydroxyapatite by bone-seeking agents offers great possibilities.

Diverse compounds such as BPs, tetracyclines, acidic oligopeptides, and oestradiol analogues have been used to target bone diseases. These compounds have the capacity to bind hydroxyapatite differentially, depending on the crystal size and intrinsic molecule modifications. As the hydroxyapatite crystal structure or exposure is disease specific (for instance, hydroxyapatite crystals in a bone tumour are different from the crystals exposed in a bone fracture), precise targeting can be achieved by using targeting molecules with selectivity for different disease states (Low and Kopecek, 2012). The binding rate of acidic oligopeptides to hydroxyapatite is faster than that of BPs but BPs have a superior binding strength and specificity for hydroxyapatite (Murphy *et al.*, 2007). BPs have the capacity to bind to all bone, while aspartate (Asp8, a common bone-targeting acidic oligopeptide) binds preferentially to higher crystalline hydroxyapatite, characteristic of resorption surfaces (Wang *et al.,* 2006). Meanwhile, tetracycline deposits preferentially onto low crystallinity hydroxyapatite that is mainly present in growing surfaces (Miller *et al.,* 2008). Altogether, when considering approaching bone mass indirectly by means of targeting the main cells responsible for bone formation (osteoblasts) and resorption (osteoclasts), osteoclasts are better targeted with acidic oligopeptides, and osteoblasts with tetracycline (Low and Kopecek, 2012). BPs, such as alendronate (Alen), are distributed to both bone-formation and bone-resorption surfaces (Wang *et al.,* 2006). More recently, (AspSerSer) 6 was found to be a targeting moiety *in vivo* for bone-formation surfaces (Zhang *et al.*, 2012a), constituting an alternative to tetracycline that presents several side effects such as teeth staining and inhibition of skeletal growth in children (Demers *et al.*, 1968; Vennila *et al.*, 2014). Several preclinical studies considering different bone diseases have tested the potential of bone-seeking agents in conjugation with nanomedicines to be used as drug delivery systems to bone (Hirabayashi and Fujisaki, 2003).

BPs are the most used bone-seeking agent in preclinical research. Different studies comprising BPs' conjugation to small molecules (Hirabayashi *et al.*, 2001a; Hirabayashi *et al.*, 2001b), linear macromolecular carriers (Wang *et al.*, 2005), proteins (Gittens *et al.,* 2005) or nanoparticles (de Miguel *et al.*, 2014; Park *et al.*, 2003; Ross and Roeder, 2011) have been performed (Hirabayashi and Fujisaki, 2003). BP-mediated bone targeting showed promising results in improving and prolonging the drug effects in different bone diseases approaches such as: prostaglandins (Gil *et al.*, 1999), oestradiol (Bauss *et al.*, 1996; Fujisaki *et al.*, 1997; Fujisaki *et al.*, 1998), synthetic estrogenic agents (Tsushima *et al.*, 2000) and nitric oxide (NO) (Lazzarato *et al.*, 2005; Nichols *et al.*, 2012; Zhang *et al.,* 2007) in osteoporosis; nonsteroidal anti-inflammatory drugs (Hirabayashi *et al.,* 2001b) in osteoarthritis; fluoroquinolones (Herczegh *et al.*, 2002) in chronic infections treatment; and cisplastin (Klenner *et al.*, 1990a); melphalan (Klenner *et al.*, 1990b), and methotrexate (Hosain *et al.*, 1996; Yang *et al.*, 2014) for the treatment of bone metastatic cancer. For further examples please see Table 2. Tetracycline and acidic oligopeptides have been

Table 2. Delivery of BP-conjugated drugs for bone diseases.

Therapeutic agent	Model of study	Delivery	Efficacy [a]	Reference
Osteoporosis				
prostaglandin E2	*in vivo*, OVX rat	intravenous	More effective in bone growth stimulant	Gil *et al*., 1999
17 beta-Estradiol	*in vivo*, OVX rat	subcutaneous	Similar effect in bone loss prevention	Bauss *et al*., 1996
	in vivo, OVX rat	intravenous	Increased bone distribution and half life	Fujisaki *et al*., 1997
	in vivo, OVX rat	intravenous	Reduction of side-effects	Fujisaki *et al*., 1998
PTH	*in vitro*, MC3T3-E1 cells	---	Increased bioactivity	Yewle *et al.*, 2013
calcitonin	*in vitro*, T47D cells *in vivo*, OVX rat	--- subcutaneous	Greater affinity and specificity for bone mineral. Improved efficacy in preserving bone volume, bone mass density and trabecular microarchitecture	Bhandari *et al.*, 2010
oestrogen (SM-16896)	*in vivo*, OVX rat	subcutaneous	Decreased efficiency in bone mass effect but reduction of side-effects	Tsushima *et al*., 2000
nitric oxide	*in vivo*, rat	intravenous	Preferential accumulation in bone. Inhibition of the differentiation of pre-osteoclasts to functional osteoclasts.	Lazzarato *et al*., 2005
Osteoarthritis				
diclofenac	*in vivo*, osteoarthritic rat	intravenous	Increased efficacy end reduction of side effects	Hirabayashi *et al*., 2001b
osteoprotegerin	*in vivo*, osteoarthritic rat	intravenous	2 and 4-fold increase in drug uptake	Doschak *et al.*, 2009
Chronic infections				
fluoroquinolones	*in vitro*, measurement of anti-bacterial activity; *ex vivo*, bone powder	---	Improved anti-bacterial activity	Herczegh *et al*., 2002
Cancer				
cisplatin	*in vivo*, osteosarcoma rat model	intravenous	Increased anti-tumour activity	Klenner *et al*., 1990a
taxanes	*in vivo*, breast cancer bone metastasis mouse model	intravenous	Enhanced anti-tumour efficacy	Miller *et al.*, 2011
	in vitro, RAW 264 and MCF-7 cell lines *in vivo*, mice with Ehrlich ascites tumour in bone	intravenous	Increased anti-tumour activity	Chaudhari *et al.*, 2012
gemcitabine	*in vivo*, mice	intravenous	Increased binding affinity to hydroxyapatite and bone	El-Mabhouh *et al.*, 2006
doxorubicin	*in vitro*, cow´s bone	---	A high binding affinity to hydroxyapatite was exhibited *in vitro*.	Hochdorffer *et al.*, 2012
proteasome inhibitors	*in vitro*, 5TGM1 and RPMI 8226 cell lines	---	Dose dependent cytotoxicity and decrease of the viable cells number	Agyin *et al.*, 2013
	in vitro, myeloma cells *in vivo*, mouse models of multiple myeloma	--- intravenous	Inhibition of myeloma growth	Swami *et al.*, 2014
arabinocytidine	*in vivo*, mouse models of tumour-induced bone disease and multiple myeloma	intravenous	Reduction in the incidence of bone metastases and overall tumour burden	Reinholz *et al.*, 2010
TNP-470	*in vivo*, K7M2 murine osteosarcoma model	subcutaneous	Increased anti-tumour activity and lower cytotoxicity	Segal *et al.*, 2011
melphalan	*in vivo*, rat Walker carcinosarcoma model combined with MNU induced mammary carcinoma	intravenous	Improved anti-carcinogenic activity	Wingen *et al.*, 1988
methotrexate	*in vivo*, rabbits	intravenous	Improved targeted delivery to bone	Hosain *et al.,* 1996
	in vitro, MG-63 human osteosarcoma cell line	---	Weaker effect in the inhibition of osteosarcoma cell proliferation.	Yang *et al*., 2014

[a] In comparison with non-conjugated drugs

Abbreviations are listed in Table 1.

used for oestradiol delivery in oedema and cancer treatment (Hirabayashi and Fujisaki, 2003) while (AspSerSer) 6 has been used in miRNA delivery for osteoporosis treatment (Ray, 2012; Zhang *et al.,* 2012a).

Concerning gene therapy, an increasing number of molecular targets and nanotechnology-based strategies are being explored (Wang and Grainger, 2012). The delivery of plasmid DNA using chitosan-based nanoparticles (Corsi *et al.*, 2003; Lu *et al.*, 2011; Zhang *et al.*, 2006) and cationic polymers (Ohashi *et al.*, 2001) has showed promising results for the treatment of joint diseases and osteosarcoma.

Alternatively, the popular RNA interference (RNAi)-based therapy using small interfering RNA (siRNA) is also exhibiting great potential both *ex vivo* and *in vivo*, to regulate progenitor cell differentiation pathways involved in osteogenic cell production for bone regeneration (Benoit and Boutin, 2012). In this regard, considering the very low cellular penetration of siRNA (consequence of its large size and anionic nature) vectors that can provide a safe and efficient gene transfer are being extensively researched. Systemic targeted-delivery strategies usually resort to known bone-homing chemistry to bind mineralised phases of inorganic bone such as the FDA approved for clinical trials, dioleoyl trimethylammonium propane-based liposomes conjugated with the targeting oligopeptide peptide $(AspSerSer)_6$ (Zhang *et al.,* 2012a). In bone repair, several gene silencing such as Noggin (Manaka *et al.*, 2011), receptor activator of nuclear factor κ B (RANK) (Wang *et al.*, 2012), NFκβ (Yao *et al.*, 2009); Src (Zheng *et al.*, 2015); osteopontin and osteocalcin (Zhang *et al.*, 2010), have been explored as a mean to regulate bone formation and mineralisation. As an example, polylactide (PLA)-polydioxanone-PEG copolymer hydrogels loaded with Noggin siRNA and bone morphogenetic protein (BMP-2) implanted into mouse dorsal pouches resulted in the stimulation of ectopic bone formation, with higher bone mineral content and greater bone quantity than that observed by delivery of an equivalent dose of BMP-2 alone (Manaka *et al.,* 2011). Layer by layer film composed of alternating layers of calcium phosphate-shRNA nanoparticles and PLL presented a sustained silencing effect for 21 d in human osteoblasts grown on the films (Zhang *et al.,* 2010). For Ewing's sarcoma (EWS), diamond nanoparticles coated with cationic polymer (nanodiamonds, NDs) have successfully been used to deliver siRNA into EWS cells where they achieved specific inhibition of EWS/Fli-1 gene expression at the mRNA and protein levels showing therefore promising data for tumour treatment (Alhaddad *et al.*, 2011; Toub *et al.*, 2006). In a different study, a polymer carrier for the delivery of siRNA to silence two distinct pathways, the PLK1 pathway for osteosarcoma cancer cells and the ERK1/2 pathway in osteoarthritis, as has been shown to slow down the progress of the diseases (Truong *et al.*, 2013).

In cell therapeutics, nanotechnology has been mainly explored for cell labelling procedures. Quantum dots, superparamagnetic and gold nanoparticles-cell labelling during regenerative therapies has been efficiently used for the visualisation and tracking of the cell transport to the area of the defect *in vivo*, and for the assessment of the fate and participation of the transplanted cells in tissue regeneration. Pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and adipocyte-derived stem/stromal progenitor cells (ASCs), are currently under extensive investigation using tissue engineering strategies for bone regeneration applications (Tautzenberger *et al.,* 2012; Vo *et al.*, 2012), namely in combination with siRNA delivery, but such approaches will not be encompassed in this review.

Targeting bone marrow cells

Together with its functions in haematopoiesis and in the immune system, bone marrow cells have also been implicated in many bone disorders such as rheumatoid arthritis, bone regeneration and repair, bone metastases and osteoporosis (Delaisse, 2014), constituting therefore a relevant target for potential therapies (Table 2).

The traditional drug delivery to bone marrow relied on uncontrolled passive diffusion through blood circulation. This is a highly ineffective method, with only a very small fraction of drugs reaching the target site. Therefore, the development of bone marrow-targeted drug delivery carriers offers great potential to the development of more efficient diagnostic and therapeutic systems. Both *in vitro* and *in vivo* tests using liposomes, polymers, microspheres, nanoparticles, silicon particles, and polypeptides as drug carriers have showed efficient targeting of bone marrow cells (Harris *et al.*, 2010; Luhmann *et al.*, 2012; Mann *et al.*, 2011; Porter *et al.*, 1992; Schettini *et al.*, 2006; Sou *et al.*, 2010; Sou *et al.*, 2011). In a study by Chi *et al.* (2010) dendritic amine and guanidinium group-modified nanoparticles were investigated for the delivery of model peptide drug into primary osteoclast precursor cells showing a significant cellular uptake of the nanoparticles (Chi *et al.*, 2010). (Porter *et al.,* 1992) showed that polystyrene particles coated with the block copolymer poloxamer-407, a non-ionic surfactant, would selectively redirect intravenously injected microspheres to sinusoidal endothelial-cells of rabbit bone-marrow (Porter *et al.,* 1992). Particles of 150 nm in diameter and below evaded recognition by Kupffer cells and efficiently accumulated in sinusoidal endothelial cells of rabbit bone marrow (Porter *et al.,* 1992). Importantly, no marked uptake was achievable with other block co-polymers having a similar structure to that of poloxamer-407, suggesting the participation of a specific interaction mechanism between the particle and the sinusoidal endothelial cell surface. After several years, the concept of active targeting to bone marrow endothelial cells was recently recharged. In a study by Chi and co-workers, dendritic amine and guanidinium group-modified nanoparticles were investigated for the delivery of model peptide drug into primary osteoclast precursor cells showing a significant cellular uptake of the nanoparticles (Chi *et al.,* 2010). Harris *et al.* prepared cationic nanoparticles with plasmid DNA and then coated their cationic surface with poly(anionic)poly(glutamic acid)-based peptides, with and without cationic insert, and showed that the terminal sequence insert, cationic amino acid sequence G-dP-dL-G-dV-dR-G, to the poly(glutamic acid)-based peptides is a critical factor for enhancing bone marrow and spleen-specificity of gene delivery *in vivo* (Harris *et al.,* 2010).

Based on the evidence that E-selectin, expressed on normal endothelial cells in response to inflammatory stimuli, is constitutively expressed in bone marrow endothelium and is involved in leukemic cell homing (Sipkins *et al.*, 2005), Mann and co-workers developed porous silicon particles modified with E-selectin thioaptamer ligands to target bone marrow endothelium (Mann *et al.,* 2011). Studies on mice demonstrated an 8-fold increase on the accumulation of porous silicon particles modified with E-selectin compared to control porous silicon particles, which were accumulated primarily in the liver and spleen instead of bone marrow (Mann *et al.,* 2011).

The microvascular bed in cortical bone consists of endothelial cells with no phago-endocytotic activity nor fenestration, and restricts the passive transvascular transport of macromolecules to particles that are able to pass ~ 5 nm pores within the endothelium. In contrast, sinusoidal blood capillaries in bone marrow are characterised by diaphragmed *fenestrae* and phago-endocytotic activity of endothelial cells (Sarin, 2010). Systemically administered particles may permeate sinusoidal endothelium and be sequestered into bone marrow, which has been explored for bone marrow imaging using radiolabelled albumin microspheres and microaggregates (10-30 nm) and polyvinylpyrrolidone nanoparticles (1-13 nm) (Moghimi *et al.*, 1990). Liposomes consisting of distearoylphosphatidylcholine (DSPC), cholesterol, PEG(5000)-DSPE, and α-tocopherol prepared in various sizes (136-318 nm diameter) have been tested for organ distribution in rabbits but none of these liposomes showed a significant accumulation in bone marrow (Awasthi *et al.*, 2003).

Overall, active and passive targeting of systemically administered particles to the bone marrow remains a challenging task. Presently, the active targeting of particles through the sinusoidal epithelia of bone marrow is a promising approach to targeted deposition of delivery systems in the bone marrow. Most studies suggested a predominant role of specific targeting over particle size for accumulation in bone marrow, but systematic studies are still required.

Local drug delivery systems

Although implicating a more invasive approach, the local delivery of drugs, entails some advantages over the systemic delivery: i) the drug quantity necessary is reduced, ii) unwanted side effects on other cells or organs/tissues is minimised, iii) drugs are retained and kept in the local for increased periods, increasing the treatment time and efficacy and iv) the adequate tuning of the delivery system allows a time-controlled delivery according to the requirements.

Ideally, local drug delivery system to bone should be designed to deliver the drug through minimally invasive procedures, such as by injection to the local site, and possess the ability for *in situ* matrix formation. In this way, wound healing would be significantly accelerated, requirements of irregular shape defects are more easily met and patients comfort and compliance is increased (Xu *et al.*, 2008; Yasmeen *et al.*, 2014). Moreover, materials should be biodegradable, able to uptake large quantities of drug, retain the drug at the desired site and release the drug slowly to the bone and surrounding tissue.

Delivery systems for the local drug release are the most investigated approach in bone therapeutics and the design options are virtually unlimited. Still, for clinical applications novel injectable biomaterials with improved properties such as mechanical strength, biocompatibility, and vascularisation are necessary.

The most investigated drugs in local drug delivery systems to bone are anti-cancer drugs, BPs and growth factors. Numerous organic, inorganic and combined systems are available for the local drug delivery into bone. Organic nanoparticles include lipids, dendrimers, chitosan, carbon nanotubes (Moore *et al.*, 2013), nanoparticles such as poly-L-lysine (PLL), PLA, polyglycolic acid, polycaprolactone (PCL) or copolymers of these such as PLGA. The inorganic nanoparticles are usually composed of silica, hydroxyapatite, or metals such as gold, silver and iron (Tautzenberger *et al.,* 2012). In Table 3 we present exemplary studies on different materials used in local delivery of therapeutics to bone.

Depending on their interaction with the host tissue, materials can be divided in bioinert and bioactive. It is now clear that besides the composite, the inherent nanostructure, namely the hierarchical porous structure, plays a major role the bioactivity level of bioactive materials. For this reason, in the past decade, synthesis and application of mesoporous solids, with highly ordered structures, high surface area and large pore volume have been significantly investigated for the encapsulation of pharmaceutical therapeutics (Vallet-Regi *et al.*, 2012). This material has showed excellent performance in both controlled drug delivery with sustained release profiles and formulation of poorly aqueous soluble drugs with enhanced bioavailability (Shen *et al.*, 2013). With the preparation of the first mesoporous bioactive glasses (MBG) in 2004 by Yan *et al*., (Yan *et al.*, 2006; Yan *et al.*, 2004), the combination of drug delivery with bioactive materials was achieved, starting a new model for improved bone regeneration applications by the functional effect of nanomaterials. These materials are based on a $CaO\text{-}SiO_2\text{-}P_2O_5$ composition and have a highly ordered mesopore channel structure with a pore size ranging from 5-20 nm. Compared to conventional non-MBGs, the MBGs have improved surface area, pore volume, ability to induce *in vitro* apatite mineralisation in simulated body fluids, and excellent cytocompatibility (Alcaide *et al.*, 2010; Li *et al.*, 2007a; Wu *et al.*, 2014; Yan *et al.,* 2006). Moreover, MBG 3D porous scaffolds can be prepared by high-throughput techniques and can, therefore, yield a highly controlled material structure to be used in bone tissue engineering and drug delivery applications (Wu and Chang, 2012). Different studies have reported an efficient therapeutics delivery to bone by means of MBG scaffolds (Wu and Chang, 2012; Wu *et al.*, 2013; Wu *et al.*, 2011).

Functional ions, such as Li, Sr, Co and B; small molecular drugs like dexamethasone, dimethyloxaloylglycine (DMOG), and gentamicin; and growth factors such as vascular endothelial growth factor (VEGF) and BMP loaded into MBG scaffolds showed great potential as a release

Table 3. Exemplary studies on different materials used in local delivery of therapeutics to bone.

	Therapeutic agent	Delivery	Therapeutic indication	Model of study	References
Inorganic					
MS and MS/apatite nanoparticles	OPG	Implant	Bone regeneration	*In vitro;* Simulated body fluid	Mendes *et al.*, 2013
CaP nanocapsules	Sim	Injection	Osteoporosis	*In vivo*; Ovariectomised mouse	Ito *et al.*, 2013
HAp	Pur	Implant	Fracture	*In vivo*; Foetal chick femur	Gellynck *et al.*, 2013
CaP cement	Alen	Implant	Osteoporosis	*In vitro*	Jindong *et al.*, 2010
Nanodiamonds	BMP-2; bFGF	Injection	Bone formation	*In vitro*, Osteoblast progenitor cells	Moore et al., 2013
CaP	Zol	Injection	Osteoporosis	*In vivo*, Ovariectomised rat and sheep	Verron *et al.*, 2010
CaS	Sim	Implant	Fracture	*In vivo*, Rat tíbia osteotomy	Qi *et al.*, 2013
CaP granules (Tetrabone®)	Helioxanthin agonist	Implant	Fracture	*In vivo*, Rat Femur bone defect	Maeda *et al.*, 2013
LDH nanocarriers	MTX	-	Osteosarcoma	*In vitro*, Saos-2 cell culture	Oh *et al.*, 2011
Ti	BMP-2	Implant	Fracture	*In vivo*, Rat tibial defect	Ishibe *et al.*, 2009
Organic					
Collagen	Ros	Implant	Fracture	*In vivo*, Critical-size rabbit bone defect	Monjo *et al.*, 2010
Collagen sponge-Teraplug®	N-acetyl cysteine	Implant	Fracture	*In vivo*, Critical-size rat femur bone defect	Yamada *et al.*, 2013
Gelatine hydrogels	rhBMP-2	Implant	Bone formation	*In vivo*, mouse subcutaneous	Kimura *et al.*, 2010
PLGA	FTY720	Implant	Allograft implants	*In vivo*, Critical-size rat tibia defect	Petrie Aronin *et al.*, 2010
PLGA/PLLA nanofibrous scaffolds	Dox	Implant	Periodontitis and RA	*In vitro*, antibacterial assays	Feng *et al.*, 2010
PLA-Nanospheres	BMP-2	-	Fracture	*In vitro*, microculture tetrazolium test	Chen *et al.*, 2011a
Polyurethane scaffolds	Los	Implant	Fracture	*In vivo*, critical-sized rat femur bone defect	Yoshii *et al.*, 2014
Organic-Inorganic					
PLGA – MS membranes (SBA15)	rhBMP-2	Implant	Bone tissue engineering	*In vitro*, human bone marrow-derived mesenchymal stem cells	Zhou *et al.*, 2014
B-TCaP / Collagen	FGF-2	Injectable	Fracture	*In vivo*, rabbit tibia segmental defect	(Komaki *et al.*, 2006)
CaP /Gelatine microparticles	TGF-ss 1	Injectable	Fracture	*In vivo*, Rabbit femoral bone defect	Link *et al.*, 2008
HAp plasma-coated Ti alloy cylinders	Zol	Implant	Implant fixation in osteoporotic bone	*In vivo* Ovariectomized sheep	Stadelmann *et al.*, 2008
PLGA/HAp microspheres	Sim	-	Osteonecrosis bone defects	*In vivo*, mouse model of gap fracture bridging with a graft of necrotic bone	Tai *et al.*, 2013
3D apatite-coated CH/CS scaffold	BMP-2, ASCs	Implant	Bone defects	*In vivo*, rat critical-sized mandibular defect model.	Fan *et al.*, 2014

Abbreviations are listed in Table 1.

system to enhance osteogenesis, angiogenesis and anti-bacterial/cancer activity (Wu *et al.,* 2014). Li, Sr, Mg, Cu, Fe, B and Zr presented a positive effect on osteogenesis. Cu and Co ions were shown to stimulate angiogenesis and Ag and Cu ions showed anti-bacterial activity. The combined release of Si ions with Li, Sr, Cu displayed a synergistic effect and a further enhancement of the osteogenesis and angiogenesis. The delivery of therapeutic drugs and growth factors, such as Dexamethasone, DMOG, BMP and basic fibroblast growth factor (bFGF) from MBG, significantly promoted *in vitro* and *in vivo* osteogenesis. DMOG and VEGF release showed an increase of angiogenesis. Gentamicin and ampicillin delivery inhibited the bacterial activity and doxorubicin presented anti-cancer effect (Wu *et al.,* 2014).

Although nanoparticles alone can provide sustained and even sequential release *in vitro* (Chen *et al.,* 2011a; Yilgor *et al.*, 2010) in bone tissue engineering applications, their incorporation into a scaffold or hydrogel matrix creates additional control over the kinetic properties, increasing treatment efficacy and specificity for improved bone repair (Isikli *et al.*, 2012; Nandagiri *et al.*, 2011). Most frequently, nanoparticles are combined with scaffolds such as solid implants, hydrogels or degradable polymeric matrixes, which can be stimulus responsive or have pre-programmed release kinetics to deliver bioactive molecules in a localised, spatiotemporal manner in agreement with the natural wound healing process. Complementary to this, the scaffold should provide support for cell migration, proliferation, synthesis extracellular matrix

and establishment of vascular networks. Properties such as injectability and *in situ* forming gels have also been highly explored in order to minimise surgical procedures (Yasmeen *et al.,* 2014). Again, a large variety of inorganic, natural, and synthetic materials have been used for the generation of controlled system scaffolds (Vo *et al.,* 2012).

Hydrogels

Hydrogels are among the most studied materials for the development of novel delivery systems for bone regeneration by combining a good biocompatibility with the capacity to provide the controlled release of functional therapeutics and a tissue-compatible substrate for cell attachment and growth, simulating an artificial extracellular matrix (Vo *et al.,* 2012).

They are a class of hydrophilic, crosslinked polymeric networks, insoluble in water that possess the ability to hold large amounts of fluids within its structure: Very different experimental settings ranging from different materials sources to the cross-linking degree, the drug-loading and release mode are being, explored with the purpose of drug delivery to bone. The most explored therapies concern protein, antibiotics and cell delivery. Both synthetic materials, such as PEG-based polymers (Lin and Anseth, 2009; Milleret *et al.*, 2014), and natural polymers like gelatine (Young *et al.*, 2005), alginate (Kolambkar *et al.*, 2011; Miao *et al.*, 2014; Rubert *et al.*, 2013), fibrin (Hong *et al.*, 2014; Yang *et al.*, 2010), and hyaluronic acid (Kim *et al.*, 2007) are being considered. Studies in a rabbit segmental defect and mouse subcutaneous implantation model propose an optimum hydrogel water content and crosslinking density for synchronised delivery and bone formation (Kimura *et al.,* 2010; Yamamoto *et al.*, 2006). Moreover, "stimuli-responsive" or "smart" hydrogels, with the capacity to respond to external stimuli, such as pH, temperature or electric stimuli are being developed (Basak *et al.*, 2014; Sood *et al.*, 2014) creating exciting new opportunities for controlled drug delivery.

Still, among their characteristics, hydrogels also possess weak mechanical properties and low bioactivity (Shaikh *et al.*, 2010) and the most recent strategies in this field are focusing in the combination of hydrogels with nanofillers, such as carbon nanotubes in a way to surpass such setbacks (Cirillo *et al.*, 2014; Yasmeen *et al.,* 2014). The development of injectable, stimuli-responsive gels containing hydroxyapatite and carbon nanotubes as nanofillers are among the latest approach in hydrogels delivery systems to bone (Yasmeen *et al.,* 2014).

Stimulus responsive

In recent years, biomaterials research as advanced from the investigation of biocompatible and biodegradable materials to the design of stimuli-responsive biomaterials that can respond to a variety of stimuli, including selective physical (*e.g.,* temperature), chemical (*e.g.,* pH and ionic strength) and biomolecular recognition (*e.g.,* enzyme, peptide- and lipid-based interactions) stimuli (Lu *et al.*, 2014). Some of these stimuli, like the pH in different cellular compartments or disease states, occur naturally *in vivo*, while others, such as light and ultrasounds, are externally controlled allowing the external temporal and spatial control of the release (Fleige *et al.*, 2012).

Considering the fact that bone repair entails a combination of sequential and cooperative signalling events in response to changes in their microenvironment the incorporation of stimulus-responsive elements into therapeutics delivery vehicles allows for improved biomimetic strategies.

Temperature and pH-sensitive polymers are among the most studied stimulus-responsive materials, mainly in combination, to form dual-responsive delivery systems. Temperature-sensitive polymers generally show low critical solution temperature behaviour, where if a certain temperature threshold is surpassed the polymer undergoes a reversible phase transition that can be exploited for drug release. By varying the hydrophilic or hydrophobic co-monomer content the low critical solution temperature can be tuned to create the desired temperature transition (Shaikh *et al.*, 2010). pH responsive polymers result from the presence of weakly acidic (*e.g.,* carboxylic and sulphonic acids) and/or weakly basic (*e.g.,* ammonium salts) functional groups on the polymeric backbone, which allow for reversible swelling/de-swelling behaviour in acidic or basic media (Shaikh *et al.,* 2010). Poly(N-isopropylacrylamide) (Garbern *et al.*, 2010; Na *et al.*, 2007), poly(organophosphazenes) (Chun *et al.*, 2009), PEG-based di/tri block copolymers (He *et al.*, 2008), PCL (Kim *et al.*, 2006) and their derivatives as well as biomimetic materials like chitosan, dextran and elastin-like peptides (Bessa *et al.*, 2010; Patois *et al.*, 2009) are the most usually temperature-responsive polymers used: Among the pH-sensitive carriers, the Poly (vinyl alcohol) and poly(acrylic acid) are amid the most common examples (Kurkuri and Aminabhavi, 2004; Zhang *et al.*, 2012b). Moreover, also pH-sensitive linkages such as hydrazone, hydrazide and acetal are extensively used for drug conjugation to a polymeric backbone (Fleige *et al.,* 2012). As an example, for bone tissue engineering, *in vivo* analysis of a pH/thermo-sensitive sulphamethazine oligomers (SMO)-poly (ε-caprolactone-*co*-lactide) (PCLA)-PEG-PCLA-SMO block copolymer loaded with human mesenchymal stem cells (hMSCs) and BMP-2 (Kim *et al.*, 2009) showed hMSC differentiation for up to 7 weeks, mineralised tissue formation and high levels of alkaline phosphatase activity. For osteomyelitis treatment (Peng *et al*., 2010a; Peng *et al.,* 2010b) developed a thermosensitive implant composed of PEG monomethyl ether (mPEG) and PLGA copolymer (mPEG-PLGA) for teicoplanin delivery. Histological staining and immunoblotting analyses in rabbits showed that the mPEG-PLGA hydrogel containing the antibiotic teicoplanin was effective in treating osteomyelitis (Peng *et al.*, 2010b). In osteoarthritis, the delivery of the anti-inflammatory celecoxib by a fully acetyl-capped PCLA-PEG-PCLA triblock copolymer hydrogel showed to be a safe drug delivery platform for sustained intra-articular release (Petit *et al.*, 2014).

In a different approach, the biochemically triggered drug release can be attained by incorporation of cleavable peptides for enzymatic degradation such as matrix metalloproteinase peptide crosslinkers (Chung *et al.*, 2006;

Fonseca *et al.*, 2014; Kim *et al.*, 2010; Lutolf *et al.*, 2003). Enzyme-cleavable peptides can also be used to connect proteins and pro-drugs for delayed release and activation in response to cell infiltration (Arrighi *et al.*, 2009; Purcell *et al.*, 2014; Wilson and Guiseppi-Elie, 2013).

Additional drug release approaches in response to magnetic, ultrasound, irradiation and electric stimuli have also been explored (Torchilin, 2014). Magnetic nanoparticles are in fact a popular approach for local bone delivery allowing a controlled, spatio-temporal release of different drugs by the use of a magnetic field after implantation *in vivo* (Akbarzadeh *et al.*, 2012; Li *et al.*, 2016). Moreover, this also permits the creation of drug gradients and long term sustained release (Sensenig *et al.*, 2012). In a study by Butoescu *et al.,* dexamethasone-containing superparamagnetic iron oxide nanoparticles (SPIONs) co-encapsulated into PLGA microparticles showed excellent biocompatibility with synoviocytes, and suggested that this type of carrier could be used as a suitable magnetically retainable intra-articular drug delivery system for treating joint diseases such as arthritis or osteoarthritis (Butoescu *et al.*, 2009). For bone tissue engineering, *in vitro* analysis of clodronate loaded hydroxyapatite-magnetite-multi-walled carbon nanotubes nanocomposite showed good results as a multimodal platform combining the effect of bone biomineralisation induced by HAp-based composites with the decrease of osteoclast formation induced by the drug (Pistone *et al.*, 2014). In a different study, three-dimensional (3D) magnetic Fe_3O_4nanoparticles containing mesoporous bioactive glass/PCL (Fe_3O_4/MBG/PCL) composite scaffolds loaded with doxorubicin as a model anticancer drug exhibited potential multifunctionality of enhanced osteogenic activity, local sustained drug release and magnetic hyperthermia *in vitro* (Jianhua Zhang, 2014).

Combined delivery

Bone healing involves the intervention of a complex and well spatio-temporal-orchestrated set of factors (Dimitriou *et al.*, 2005; Tsiridis *et al.*, 2007). In this regard, the incapacity to mimic the complex tissue architecture and to provide the necessary biochemical and cellular microenvironment is a major challenge in tissue regeneration.

Studies have shown that sequential and spatiotemporal drug release may lead to improved tissue regeneration by providing physiologically relevant release profiles and spatial gradients that mimic the natural healing response (Lee *et al.*, 2011; Santo *et al.*, 2013; Vo *et al.,* 2012). The designing of multi-functional nanomaterials that enable the simultaneous, sequential and spatially-controlled delivery strategies of different therapeutics have been developed to recapitulate the early expression of factors for bone regeneration.

Several *in vitro* and *in vivo* studies have investigated the synergistic effects of dual growth factor delivery (BMP-2, BMP-7, VEGF, transforming growth factor (TGF)-β-3, Insulin-like growth factor 1 (IGF-1)) using different scaffold composites such as alginate, periodontal ligament fibroblasts, PLGA, gelatine and nanodiamonds, for numerous bone and cartilage regeneration applications (Basmanav *et al.*, 2008; Chen *et al.*, 2009; Facca *et al.*, 2010; Kanczler *et al.*, 2010; Moore *et al.,* 2013; Park *et al.*, 2009; Patel *et al.*, 2008; Richardson *et al.*, 2001; Simmons *et al.*, 2004) showing improved results in comparison with the single delivery. In a recent study, the simultaneous delivery of BMP-2 and bFGF by nanodiamonds has showed good results for the promotion of bone cell differentiation and proliferation *in vitro* (Moore *et al.,* 2013).

Combined delivery of growth factors and drugs has also been addressed as shown in a study of Choi *et al.,* where a dual and time-controlled release of BMP-2 and dexamethasone from PLGA-core/alginate-shell microcapsules fabricated with coaxial electro-dropping was investigated (Choi *et al.*, 2010). In the past 5 years, MBGs have also been largely investigated for the combined delivery of therapeutic ions and drug/growth factors (Wu and Chang, 2014). Still, all *in vivo* reports of enhanced bone formation upon combined delivery of growth factors have been performed in ectopic, but not orthotopic models and further studies are necessary.

In a different approach, the time-dependence requirements of delivery have been investigated. Gelatine microparticles-loaded poly (propylene fumarate) scaffolds showed that bone formation in a rat calvarial critical size defect was BMP-2 dose-dependent and that VEGF combined delivery was beneficial on bone regeneration at 4 weeks, but not at 12 weeks (Patel *et al.,* 2008; Young *et al.*, 2009). Also, the sequential release of VEGF and BMP-2 from PLGA microsphere-loaded poly(propylene fumarate) scaffold-gelatine hydrogel composites in a rat femoral defect over 8 weeks showed suboptimal bone formation (Kempen *et al.*, 2009).

In order to mimic the physiological concentration gradients that provide the topological signals for tissue formation, the impact of spatial delivery has also been addressed. Silk fibroin microspheres immobilised in a 3D porous silk scaffold where used for the simultaneous release of linear gradients of recombinant human BMP-2 and IGF-1 – showing enhanced osteogenic and chondrogenic differentiation of hMSCs over 5 weeks along the gradients (Wang *et al.*, 2009).

Multilayered scaffolds constitute a unique platform that enables multiple delivery of therapeutics through incorporation of different phases into scaffolds for differential release kinetics and spatially controlled drug release. Bi-layered oligo(poly(ethylene glycol) fumarate) and gelatine microparticles hydrogels developed by (Guo *et al.,* 2009; Guo *et al.,* 2010) showed enhanced chondrogenic differentiation of encapsulated rabbit MSCs *in vitro* with delivery of TGF-β1 or TGF-β3 in the chondrogenic layer and co-culture with pre-differentiated osteogenic cells in the lower bone-forming layer (Guo *et al.*, 2010; Guo *et al.*, 2009). More recently, the potential of layer-by-layer technique has been successfully applied for the development of multilayered coating scaffolds for controlled drug delivery for orthopaedic implant applications (Kunjukunju *et al.*, 2013; Min *et al.*, 2014).

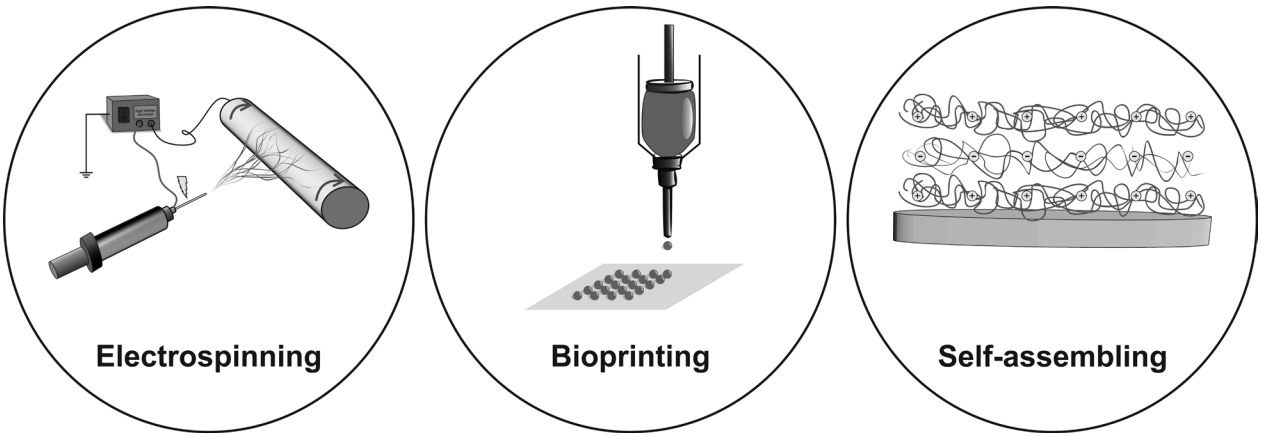

Fig. 2. Schematics of **a**) Electrospinning, **b**) Bioprinting, and **c**) Layered Scaffolds processing techniques.

High-throughput processing techniques

Manufacturing techniques like electrospinning, bioprinting, and layered scaffolds/membranes (Fig. 2), are among the latest techniques being pursued to produce tissue constructs and engineered scaffolds with pre-designed macro- micro- and nano- features, gradient physic-chemical properties and custom shapes for tissue engineering therapies.

Electrospinning is a technique that produces fibrous scaffolds and films through electrostatic repulsion of liquid polymer solutions using a high-voltage source creating nano/micron-sized fibres suitable for cell attachment and growth. Their orientation and geometries for therapeutics release can be controlled by collecting the extruded fibres on various static or rotating plates. Several studies reported sustained release of therapeutics to bone from electrospun scaffolds (Dong *et al.*, 2014; Lee *et al.*, 2014; Mi *et al.*, 2014; Ramier *et al.*, 2014; Ren *et al.*, 2014; Sahoo *et al.*, 2010; Srouji *et al.*, 2011; Tetteh *et al.*, 2014). As an example, in a study by Sahoo *et al.,* PLGA nanofibers incorporated with bFGF, fabricated using the facile technique of blending and electrospinning (Group I) and by the more complex technique of coaxial electrospinning (Group II) presented prolonged growth factor release that positively influenced stem cell behaviour and fate (Sahoo *et al.,* 2010).

Moreover, exciting new technology such as biopatterning offers a more precise and efficient control of drug delivery than conventional techniques. Biopatterning is a promising approach in bone tissue engineering, used to immobilise growth factors and cells onto 3D scaffolds with persistent patterns that subsequently control cell migration, proliferation and differentiation. In this technique a precise positioning of biological materials, biochemicals and living cells, with spatial control of the placement of functional components, is used to fabricate 3D structures (Guillemot *et al.*, 2010). Cooper *et al.* used acellular-derma-matrix constructs patterned with BMP-2 and signalling noggin protein implanted into a mouse calvarial defect model showing that three-dimensional biopatterning of a growth factor and growth factor modifier within a construct can direct cell differentiation *in vitro* and tissue formation *in vivo* according to printed patterns (Cooper *et al.*, 2010). In a similar study, Herberg *et al.* showed that sustained release delivery of a low-dose growth factor therapy (BMP-2, stromal cell-derived factor-1β and TGF-β1), and co-therapies using inkjet-based biopatterning of acellular-derma-matrix can aid healing in mouse calvarial critical size defect (Herberg *et al.*, 2014). Gao *et al.* evaluate bioactive ceramic nanoparticles (glass (BG) and hydroxyapatite) in stimulating osteogenesis of printed bone marrow-derived hMSCs in poly(ethylene glycol) dimethacrylate scaffold. Results highlighted the technology capacity cell delivery into hard tissue engineering with biomimetic structures (Gao *et al.*, 2014). This strategy enables the design of spatially customised anatomical architecture in a patient- and defect-specific manner.

Layer-by-layer assembly of nanoparticles and coatings allows for the modification of the material´s surface properties, enabling a controlled release of desirable substances (Min *et al.,* 2014). By using this technique, stronger bonding of implants to bones can be achieved as well as the controlled release of antibiotics or antimicrobial agents to prevent infection (Gaudiere *et al.*, 2014; Hu *et al.*, 2010; Li *et al.*, 2012; Shah *et al.*, 2013; Yang *et al.*, 2009).

A list of currently ongoing approaches for the biomaterial-based delivery of therapeutics for different bone diseases is listed in Table 4.

***In silico* mathematical models for the skeletal system**

Mathematical modelling has been shown to be an important tool in the optimisation of drug delivery systems (Siepmann and Siepmann, 2008). Advanced modelling and *in silico* experiments can be useful in different components of the optimisation process, such as calculation of optimal drug release profiles (Raiche and Puleo, 2001) specification of structure and dimensions of delivery systems (Velghe *et al.*, 2014), or to speed up product development (Siepmann and Siepmann, 2012), just to name a few.

Mathematical and computational models provide detailed quantitative descriptions of a system by capturing, in one or more mathematical expressions, its main dynamics and properties. Models are, of course, an abstraction of the real systems, with underlying simplifications and assumptions, but have the potential to isolate and quantitatively describe the mechanisms of

Table 4a. Biomaterial-based delivery of therapeutics for bone diseases.

Therapeutic agent	Biomaterial	Delivery	Model of study	References
Osteoporosis				
Sim	CaP nanocapsules	injection	*in vivo*, ovariectomised mouse	Ito *et al*., 2013
Alen	CaP cement	implant	*in vitro*, rat mesenchymal stem cells	Jindong *et al*., 2010
Zol	CaP	injection	*in vivo*, ovariectomised rat and sheep	Verron *et al*., 2010
	HAp plasma-coated Ti alloy cylinders	implant	*in vivo*, ovariectomised sheep	Stadelmann *et al*., 2008)
PTH 1-34	CH NPs	multiple routes	*in vitro*, Saos-2 cells	Narayanan *et al.*, 2012
	microneedle patch system	transdermal	phase 2 clinical trials	Daddona *et al.*, 2011
Zol +bFGF	HAp-coated Ti implant:	implant	*in vivo*, ovariectomised rats	Gao *et al.*, 2009
rhBMP-7	PLGA NS onto nano-fibrous PLLA scaffolds	implant	*in vivo, rats*	Wei *et al.*, 2007
Oestrogen	CaPC	implant	*in vivo*, rats	Otsuka *et al.*, 2000
Ris	polymeric NPs	intranasal	*ex vivo*, porcine fresh nasal mucosa; and *In vivo,* aged rats	Fazil *et al.*, 2015
siRNA (Plekho1)	(DOTAP)-(AspSerSer) (6) liposomes	injection	*in vivo,* osteoporotic rats	Zhang *et al*., 2012a
Plasmid DNA	CH	---	*in vitro,* MSCs, MG63 and HEK293	Corsi *et al*., 2003
Fractures				
FTY720	PLGA-coated demineralised allograph	implant	*in vivo*, critical-size rat tibial defect	Petrie Aronin *et al*., 2010
Pur	HAp	implant	*in vivo*; foetal chick femur	Gellynck *et al*., 2013
Sim	CaS	implant	*in vivo*, rat tíbia osteotomy	Qi *et al*., 2013
	PLGA/HAp microspheres	local administration	*in vivo*, mouse model of gap fracture bridging with a graft of necrotic bone	Tai *et al*., 2013
Lov	Polyurethane scaffolds	implant	*in vivo*, critical-sized segmental defect in rat femora	Yoshii *et al*., 2014
Ros	Collagen sponge	implant	*in vivo*, critical-size rabbit cortical bone defect	Monjo *et al*., 2010
Helioxanthin	CaP granules (Tetrabone®)	implant	*in vivo*, Rat Femur bone defect	Maeda *et al*., 2013
BMP-2	PLA-NPs	not disclosed	*in vitro*, microculture tetrazolium test	Chen *et al*., 2011a
FGF-2	B-TCP / Collagen	injectable	*in vivo*, rabbit tibia segmental defect	Komaki *et al*., 2006
TGF-ss 1	CaP /Gelatine microparticles	injectable	*in vivo*, rabbit femoral bone defect	Link *et al*., 2008
BMP-2, ASCs	3D apatite-coated CH/CS scaffold	implant	*in vivo*, rat critical-sized mandibular defect model.	Fan *et al*., 2014
BMP-2	Heparin/apatite-coated implant	local implant	*in vivo*, rat tibial defect	Ishibe *et al*., 2009
siRNA (Noggin)	PLA-DX-PEG hydrogel	implant	*in vivo*, mouse dorsal muscle pouche	Manaka *et al*., 2011
shRNAs (osteopontin or osteocalcin)	Multi-shell calcium phosphate NPs incorporated into a PLL multilayered film	---	*in vitro,* human osteoblasts	Zhang *et al*., 2010
NO	CH	local implant	*in vivo,* rat femur fracture model.	Diwan *et al.*, 2000
NO	Demineralised bone matrix	local implant	*in vivo,* rat femur fracture model	Baldik *et al.*, 2002

Abbreviations are listed in Table 1.

interest and its causal dependencies. Two key concepts underlie the use of mathematical models in the optimisation of drug delivery systems, as in other research fields: 1) hypothesis testing – assess if a conceptual model is in fact a proper description of the mechanism(s) of interest in the real system; and 2) prediction – given partial information about the state and parameters of the system, be able to infer unknown variables (usually prediction is used in the context of producing estimates for variables of interest at future time instants). Both situations have been used in the quest to better understand and optimise the drug delivery systems (Peppas, 2013; Siepmann and Peppas, 2001a; Siepmann and Siepmann, 2008).

In order to capture and predict the spatiotemporal properties of drug delivery, existing mathematical models focus on the following mechanisms associated with the delivery system: diffusion, swelling, erosion and drug binding affinity (Arifin *et al.*, 2006; Lauzon *et al.*, 2012; Siepmann and Siepmann, 2008). While for presentation purposes the following section addresses each one of these mechanisms separately, in practise most models blend together these theories in order to produce more realistic descriptions of the drug delivery systems. This means that different types of scaffolds developed for drug delivery typically require more than one of the above mentioned core mechanisms to be properly modelled. Mathematical models, independently of the mechanism they try to describe, can either be empirical or mechanistic depending on the level of simplification used: mechanistic models include an explicit representation of mass transport mechanisms and biochemical reactions (Korsmeyer *et al.*, 1986a); empirical models try to directly capture the

Table 4b. Biomaterial-based delivery of therapeutics for bone diseases.

Therapeutic agent	Biomaterial	Delivery	Model of study	References
Bone Cancer and bone metastasis				
Ris	PLL-CD	subcutaneous injection	*in vivo*, BALB/c nu/nu mice injected with CHO-β_3 tumour cells	Daubine *et al.*, 2009
Zol -Docetaxel	PLGA-PEG	intravenous injection	*in vitro* (MCF7 and BO2 cell lines) and *In vivo* (Swiss mice with induced femoral tumour)	Ramanlal Chaudhari *et al.*, 2012
Alen	PLGA	systemic administration	*in vitro*(human venous blood, HUVECs, and human primary osteoblasts)	Pignatello *et al.*, 2009
Dox	PLGA	systemic administration	*in vivo*, orthotopic mouse model of breast cancer bone metastases	Salerno *et al.*, 2010
	alpha-AL-HPLN	systemic administration	*in vitro*, osteosarcoma cell lines	Federman *et al.*, 2012
	Lipid-modified dextran based polymeric NPs	-----	*in vitro*, osteosarcoma cell lines	Susa *et al.*, 2009
	Dextran-PEI NPs	-----	*in vitro*, Osteosarcoma cell lines	Sun *et al.*, 2011
PTX-PEG-Alen conjugate	PTX and Alen	systemic injection	*in vitro*, PC3 cells.	Clementi *et al.*, 2011
20(S)-Camptothecin	Cyclodextrin-based polymer	intravenous injection	*in vivo*, mouse xenografts cancer models	Schluep *et al.*, 2006
MTX	LDH	---	*in vitro*, Saos-2 cell line	Oh *et al.*, 2011
ATO	Magnetic nanoparticles encapsulated by PLA.	intravenous injection	*in vivo*, mouse osteosarcoma tumour models	Li *et al.*, 2007b
Cisplatin	CaP Nps	---	*in vitro*, K8 clonal murine osteosarcoma cell line	Barroug *et al.*, 2004
siRNA (FLi1 gene)	PEI and PAH coated ND.	---	*in vitro*, Ewing sarcoma cells	Alhaddad *et al.*, 2011
siRNA (PLK1 gene)	Diblock copolymer	---	*in vitro,* osteosarcoma cell line	Truong *et al.*, 2013
Arthroplasties				
rhBMP-2	Carbon nanotubes	implant	*in vivo*, mouse	Usui *et al.*, 2008
BMP-2	CH-tripolyphosphate (CH-TPP) nanoparticles	implant	*in vivo*, mouse	Poth *et al.*, 2015
Osteoarthritis				
Osteoarthritis drug	nanoparticles-in-microspheres	intra-articular injection	*in vivo*, rat model	Chen *et al.*, 2014
Dextran	Polymeric hydrogel	intra-articular injection	*in vivo*, rat model	Morgen *et al.*, 2013
Plasmid DNA	CH NPs	intra-articular injection	*in vivo*, osteoarthritis rabbits	Zhang *et al.*, 2006
Plasmid DNA	HA-CH	---	*in vitro,* chondrocyte cell culture	Lu *et al.*, 2011
siRNA (ERK1/2 gene)	Diblock copolymer	---	*in vitro,* osteosarcoma cell line	Truong *et al.*, 2013
Infectious and inflammatory bone diseases				
Dox	3D PLGA NS incorporated into prefabricated nanofibrous PLLA scaffolds	---	*in vitro*, antibacterial tests	Feng *et al.*, 2010
Teicoplanin	mPEG-PLGA termosensitive hydrogel	local injection	*in vivo*, rabbit osteomyelitis model	Peng *et al.*, 2010a
Tigecycline	CaP-PLGA NPs	implant	*in vivo*, rat	Ignjatovic *et al.*, 2010
Small molecule drug model, fluorescein	HAp/CH particles	----	*in vitro*, MC3T3-E1 cells	Uskokovic and Desai, 2014
Antibiotic	CPs	----	*in vitro*, MC3T3-E1 cells	Uskokovic and Desai, 2013
CFS	Micro- to macro-porous hydroxyapatite scaffolds	implant	*in vivo*, rabbit tibia osteomyelitis animal model and *In vivo* human bone.	Bhattacharya *et al.*, 2013
VCM	bioactive glass ceramics	-----	*in vitro*, rat osteoblastic-like cells	Thanyaphoo and Kaewsrichan, 2012
NO	xerogel-coated implant pins	implant	*in vivo,* rat	Holt *et al.*, 2011

Abbreviations are listed in Table 1.

drug release profiles without explicitly stating the internal generating mechanisms (Higuchi, 1961; Peppas, 1985).

While mathematical and computational models of drug delivery is a field in rapid development, very few studies exist in the context of the skeletal system (Lauzon *et al.,* 2012; Lauzon *et al.*, 2014; Makarov *et al.*, 2014; Raiche and Puleo, 2001).

Models for diffusion controlled systems

Mechanistic models for diffusion controlled systems are grounded in the principle of mass conservation. This principle states that in a closed system, total mass is conserved: it can neither be created nor destroyed, but it can be rearranged in space (through the action of fluxes). Given its dependence in both space and time, mechanistic models for diffusion controlled systems need to be described by partial differential equations, relating the drug concentration (*C*), with transport mechanisms (flux, *F*) and the existing drug sources and sinks (*f*):

$$\frac{\partial C}{\partial t} + \vec{\nabla} \cdot \vec{F} = f \qquad 1$$

The symbol $\vec{\nabla}$ represents the mathematical operator divergence. The physical significance of the divergence of the flux *F* can be seen as the rate at which drug density exits a given region of space (Fig 3). Each of the variables *C*, *F* and *f* may be functions of both time and space.

In the absence of transport mechanisms other than diffusion, the flux is given by Fick's first law:

$$\vec{F} = -D\nabla C \qquad 2$$

where *D* is the diffusion coefficient (which may also be a function of time and space). Initial conditions, geometry and boundary conditions all play a crucial role in the solutions of the mass conservation equation. One of the most frequently used approaches is to discard interactions of the active molecules with the environment and consider only the pseudo steady-state (Higuchi, 1961; Siepmann and Peppas, 2011). Other models have, however, considered less simplified conditions (Makarov *et al.,* 2014; Siepmann and Siepmann, 2012; Tzafriri *et al.*, 2005) including different geometries (Grassi and Grassi, 2005; Helbling *et al.,* 2011; Raiche and Puleo, 2001), moving boundaries (Manitz *et al.*, 1998), hydrogel network design (Lin and Metters, 2006), drug dissolution (Siepmann and Siepmann, 2013), and vesicle design (Mosley *et al.*, 2013).

Models for swelling controlled systems

Some polymer matrix delivery systems are subject to polymer swelling with important consequences in the properties and dynamics of drug release. This swelling affects transit times and diffusion properties by increasing the volume and changing the properties of the medium where the flux is taking place. Altering the diffusion pathways, and subsequently the concentration gradients, can produce significant changes in the drug release properties (Siepmann and Siepmann, 2008). Models dealing with swelling are therefore more complex than simple diffusion models, as they have to account for changing geometries and/or boundary conditions as well as inhomogeneous diffusion coefficients (Korsmeyer *et al.,* 1986a; Korsmeyer *et al.*, 1986b; Siepmann and Peppas, 2001b).

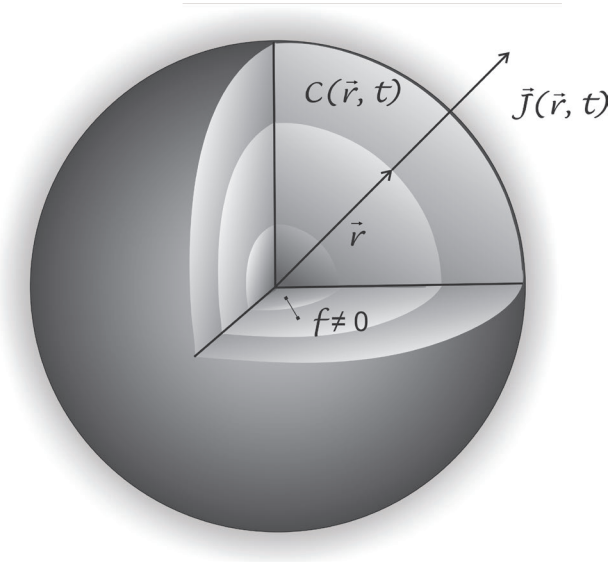

Fig. 3. Principle of mass conservation in a system with spherical symmetry. The nanoparticles/drug concentration, as a function of time and distance to the delivery system core, is described by the present fluxes *J* (*e.g.,* diffusive) and the delivery and degradation dynamics, captured in the component *f*.

Models for erosion controlled systems

Polymer erosion and degradation are other properties that can be carefully engineered to enhance tissue treatment. In the case of bone repair, generally the scaffolds must be resorbed to provide efficient healing (Lauzon *et al.,* 2012). The process of erosion/degradation can be modelled as a combination of stochastic hydrolysis and mass transport (Chen *et al.*, 2011b), autocatalytic hydrolysis (Ford Versypt *et al.*, 2013), diffusion and chemical reactions (Siepmann and Gopferich, 2001). An extensive review of different mathematical models addressing polymer erosion of the context of drug delivery is presented in (Sackett and Narasimhan, 2011).

Models for affinity controlled systems

In some delivery systems the interactions between drug and scaffold need to be accounted for in order to produce a plausible and realistic model of the drug release. In these models the mass conservation equation together with Fick's first law are used in combination with biochemical (kinetics) equations describing the affinity interactions (Fu *et al.*, 2011; Lauzon *et al.,* 2012; Lauzon *et al.,* 2014).

Conclusions and future perspectives

The relevance of nanobiotechnology in the improvement of drug delivery systems is now unquestionable and assumes a central role in the progression of human therapeutics.

Recent research has clearly demonstrated the potential of nanotechnology in the development of drug delivery systems to a vast range of bone disorders. Present pharmaceutical efforts are shifting from the search for new drugs into the investigation of novel approaches that enable a specific and targeted delivery of therapeutics to bone that may improve drug efficiency and patient compliance, while minimising adverse side-effects. Targeted delivery systems based on bone seeking agents resulted in enhanced distribution of therapeutic agents to bone tissue. Local administration based on nanoparticles and hydrogels that slowly released incorporated drugs presented a sustained therapeutic effect in disease site and most recently, smart drug delivery systems that respond to various stimuli have been investigated to attain the controlled and site-specific delivery of drugs. Complementary, diverse high throughput technologies and *in silico* approaches are currently facing a steep development and improvement. By enabling the precise processing of materials and the establishment of very fine mathematical formulation, comprising highly tuneable/case-specific parameters, such technologies represent a very exciting step forward for the achievement of the desired personalised drug delivery approach.

Although offering great potential, significant challenges and limitations in this field must be addressed in order to make drug delivery systems to bone an effective clinical reality. One of the major handicaps is the lack of knowledge of the pathological and biochemical pathways involved in specific disease states. A deeper understanding of the disease-specific underlying mechanisms and time frames would allow the design of more accurate tailored systems with enhanced effectiveness and the development of precise synergistic drug delivery systems to bone.

Nanobiotechnology-based drug delivery strategies to bone have a very wide range of applications and represent an exciting and very promising field of research with the potential to significantly improve the quality of life for millions of people.

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Discussion with Reviewers

Christine Hartmann: What are the current technical limitations to nanotechnology?

Authors: Nanotechnology has revolutionised scientific research and is presently the major driving force behind industrial and biomedical discovery. Its fields of applications are virtually unlimited and encompass enormous socioeconomic gains. Still, also potentially great putative risks must be foreseen and addressed by the scientific and governmental community. Risk assessment and risk management programs must be fostered and the exploitation of matter and processes at the nanoscale must be regulated. Currently, research on nanomaterials safety is a very hot topic and the object of significant efforts and investment as exemplified by being one of the main foci of the EU's research and innovation funding programme, Horizon 2020.

In the biological and biomedical field, a major technical hurdle is the absence of imaging equipment with detection limits within the nano range that allow a precise assessment of nanoparticles biodistribution and metabolism in live organisms.

Current bioimaging analysis require the accumulation of nanoparticles and most investigation techniques resort to *ex vivo* evaluations that are very limiting not only in the course of research but most particularly when considering the translation from animal to human applications.

Although in the last decades tremendous technological advances have been put to use in the field of nanotechnology, the understanding and control of matter and processes at the nanoscale is still far from being achieved. The lack of adequate mathematical models that describe the properties of matter at the nanoscale level is presently an important limitation for the development of novel applications that exploit the particularity of nano-materials, devices and systems in the benefit of society.

Christine Hartmann: Where do you see the greatest potential to further improve nanotechnology as a delivery system to bone?

Authors: In recent decades, the application of nanotechnology into the biomedical field has allowed an exceptional improvement of diagnostic tools and clinical treatments offering new means to overcome many of the classical drug delivery limitations, such as poor solubility, high toxicity, lack of specificity, degradation and short half-life after administration.

In bone regeneration, nanoparticle technology showed very promising results for delivery of therapeutic agents (proteins, drugs, genes) and for the design of scaffolds with improved biocompatibility and mechanical properties. Still, there is yet a long way to go before nanotechnology as a delivery system to bone becomes a clinical practice and assumes a leading role in bone regeneration therapies. Current bone-targeting delivery systems distribute drugs to the whole skeleton and not to the specific functional cells in bone, which reduces the treatment efficiency and bears the risk of toxic effects on non-targeted cells. Future efforts in the design of drug delivery systems to bone must address with precision the particularities of the bone regeneration process, such as the fact that it involves a large number of different cell types and the participation of many intracellular and extracellular signalling pathways, that interact in a space and temporal-dependent manner. A better understanding of the cellular and molecular interaction between nanostructures and the specific cell types (such as mesenchymal stem cells, osteoblasts, osteocytes, osteoclasts and even inflammatory cells) involved in bone regeneration in a time- and space- dependent manner, as well as the understanding of the underlying biochemical pathways being activated in different disease states, offers exciting new possibilities to the improvement of the targeting specificity and efficacy of delivery systems to bone and can provide a new paradigm for the treatment of bone diseases.

Editor's Note: Scientific Editor in charge of the paper: Christine Hartmann.