TISSUE ENGINEERING: Part B
Volume 18, Number 5, 2012

DOI: 10.1089/ten.teb.2011.0737

Bisphosphonate-Based Strategies for Bone Tissue Engineering and Orthopedic Implants

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Bisphosphonates (BPs) are a group of well-established drugs that are applied in the development of metabolic bone disorder-related therapies. There is increasing interest also in the application of BPs in the context of bone tissue engineering, which is the topic of this review, in which an extensive overview of published studies on the development and applications of BPs-based strategies for bone regeneration is provided with special focus on the rationale for the use of different BPs in three-dimensional (3D) bone tissue scaffolds. The different alternatives that are investigated to address the delivery and sustained release of these therapeutic drugs in the nearby tissues are comprehensively discussed, and the most significant published approaches on bisphosphonate-conjugated drugs in multifunctional 3D scaffolds as well as the role of BPs within coatings for the improved fixation of orthopedic implants are presented and critically evaluated. Finally, the authors' views regarding the remaining challenges in the fields and directions for future research efforts are highlighted.

Introduction

The interest in bone tissue engineering (BTE) and regeneration therapies is constantly growing in parallel with the rise in trauma victims and musculoskeletal disorders associated with the increase in life expectancy.[1] Engineering of bone tissue has the potential to tackle the bone lost that occurs, for example, due to degenerative, surgical, or traumatic processes.[2] In addition, there is the need to accelerate the healing of large bone fractures and to treat established nonunion problematic fractures.[2] In this context, a variety of therapeutic drugs are being evaluated in combination with tissue-engineering approaches for bone regeneration.[3,4] This review article is focused on the application of bisphosphonates (BPs) in BTE. BPs are well-established drugs that are used in the development of metabolic bone disorder-related therapies, such as osteoporosis and Paget's disease, tumour-induced hyperkalaemia, and inflammation-related bone loss.[5–7] Although the use of BPs for BTE is in its initial steps, increasing evidence on the advantages of BPs in combination with scaffolds in tissue-engineering strategies is emerging and the related literature is growing, which has prompted the preparation of this review. A brief summary of the mechanism and pharmacodynamics of BPs is provided in the first part of this review. The rationale for the use of different BPs in three-dimensional (3D) scaffolds is discussed in the next section; special emphasis is placed on the different issues that need to be addressed for the delivery and sustained release of these therapeutic drugs during the bone formation stage. In the third and the forth parts,the strategies proposed using bisphosphonate-conjugated drugs in multifunctional 3D scaffolds and the role of BPs within coatings for the improved fixation of orthopedic implants are respectively outlined. In the last section, the remaining challenges in the field and directions for future research efforts from the authors' perspective are highlighted.

Bisphosphonates

BPs are a type of drugs that are considered stable analogs of pyrophosphate, a physiological regulator of calcification and bone resorption,[8–10] in that the P-O-P bond of pyrophosphate is replaced by a P-C-P bond, which is resistant to chemical and enzymatic hydrolysis. Figure 1 shows the chemical structure of BPs in the inner circle. The R_1 and R_2 side chains attached to the carbon are responsible for the variation in activity observed among these drugs.[11] Substitutions in the side chains lead to the synthesis of a large number of compounds with different properties (Figure 1). It has been reported that R_1 groups are responsible for the targeting and binding of BPs to bone, while R_2 ones are responsible for their potency and their action on bone resorption.[12] Several

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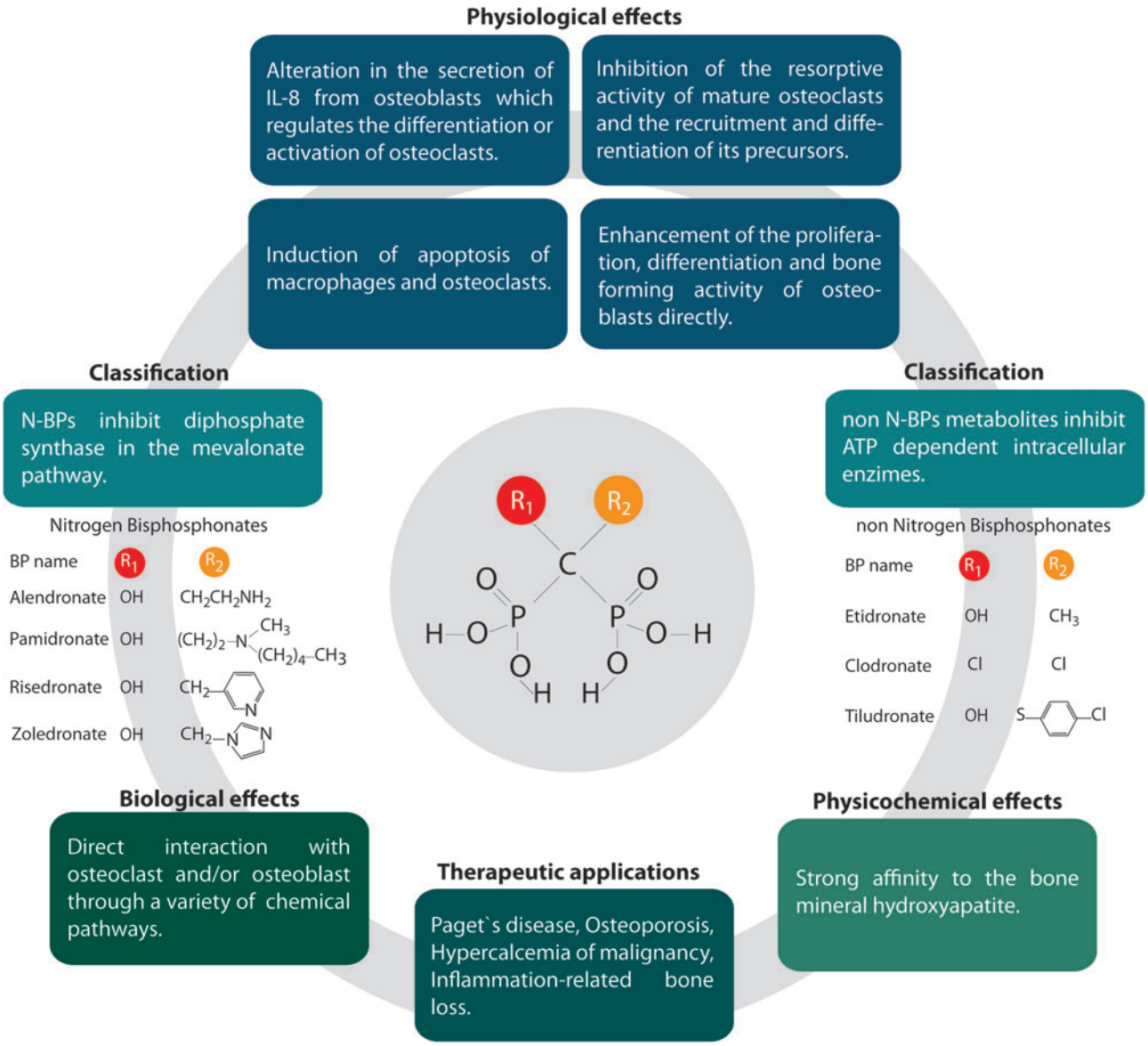

FIG. 1. This figure shows the chemical structure of bisphosphonates (BPs) (centre), the classification of BPs, and the mechanism of action depending on the classification group (sides), their physiological (top) and physicochemical (bottom, right) effects in bone tissue, as well as BPs' therapeutic applications (bottom, left).[10,16–18,48–50,57–59] Color images available online at www.liebertonline.com/teb

studies have shown that BPs with–OH and–NH_2 substitution in R_1 increase their binding to bone mineral.[13–15] BPs are classified into two groups: the Nitrogen-BPs (N-BPs), such as alendronate (ALN), residronate, ibandronate, pamidronate, and zoledronic acid, and the non-N-BPs, such as clodronate (CLO) and etidronate (Fig. 1). The classification of BPs into two groups is also according to their different mechanisms of action. The N-BPs act on the cholesterol pathway by inhibiting diphosphate synthase in the mevalonate pathway,[16,17] while the non-N-BPs are metabolically transformed into cytotoxic ATP analogs that inhibit ATP-dependent intracellular enzymes.[18] Various BPs have already been used for the clinical treatment of Paget's disease, osteoporosis, hyperkalaemia of malignancy, osteogenesis imperfect, and inflammation-related bone loss, to promote fracture repair.[5–7,19–24] The clinical pharmacology of BPs revealed that their affinity to bone mineral hydroxyapatite (HA) is the basis for their use as inhibitors of ectopic calcification and bone resorption.[10] Substantial literature regarding the pharmacodynamics of BPs also reporting on clinical results is available.[25–28] All BPs are characterized by their low bioavailability via oral administration and their associated side effects as well as adverse reactions related with parenteral administration.[10,30] Since 2003, many publications have reported an association between bisphosphonate therapy and osteonecrosis of the jaws.[166–172] Most precisely, a correlation between the types of bisphosphonate, the period of the treatment, and the occurrence and gravity of osteonecrosis of the jaws has been proposed.[167] Most of the reported cases corresponded to intravenous therapy with high doses of amino-group-containing BPs such as zoledronic acid or pamidronate for malignant disease. In addition, a few cases of osteonecrosis of the jaws corresponded to long-term

oral administration of nonaminogroup-containing BPs.[167] The mechanism behind this osteonecrosis of the jaws remains unclear. It can be speculated that with comparatively much lower doses of BP locally released—only in the required area and for a short period of time—none of the side effects just mentioned should be observed. BPs administration *in situ*, by mean of a drug delivery system, has the potential to obtain high specific bone response and optimal bioavailability, as bone targeting will concentrate the therapeutic agent at the desired site of activity. Nevertheless, it is important to highlight the importance of taking these effects into account during the evaluation of any new system of controlled local release of BP. During the last decade, several studies that are related to the development of delivery systems for the controlled release of BP have been published.[7,29,31–41] Smart methods of local drug delivery systems could increase the drug activity and specificity by concentrating the release of BP to bone sites and could reduce side effects at extra-skeletal sites. For those interested in further reading, a recent review summarizes the latest advances in the application of BPs for *in situ* orthopedic medication and treatments.[29]

BPs role in bone tissue

The bone is an essential tissue that provides protection, support, and storage of calcium and other trace inorganics ions as well as a site for production of white blood cells for the body. This mineralized tissue contains cell types of osteoblasts (bone matrix-producing cells), osteocytes (mature osteoblasts that are embedded in the mineralized matrix), osteoclasts (bone matrix-degrading cells), and osteoprogenitors (immature cells that are capable of differentiating into osteoblasts which are found in the bone marrow and periosteum). Trabecular bone (or cancelous bone) constitutes 20% of the total adult bone tissue and has a spongy branch-like structure of trabeculae, with a porosity of 50%–90%.[42–44] Cortical (or compact) bone comprises the final 80% of adult bone tissue; it is denser than trabecular bone with only 10% porosity, this makes it much more resistant to mechanical forces. The bone is a specialized connective tissue that is made up of fibrous (collagen I) and solid components (HA crystals), making it a truly composite material with a highly hierarchical structured matrix.[45,46] Collagen and HA combined in the nanoscale contribute to both the compressive strength and toughness of bone. Bone is not a static material but is constantly undergoing a state of remodeling. It secretes signaling molecules that are embedded in the matrix; when the matrix is degraded or stressed, these biomolecules are released, for example, bone morphogenic factors, which guide the remodeling process.[38] BPs are selectively taken up by osteoclasts in bone matrix containing HA and have various direct effects at the cellular level.[47] For example, BPs inhibit the recruitment and differentiation of osteoclasts precursors[48] and the resorptive activity of mature osteoclasts.[49] In addition, BPs induce apoptosis of macrophages and mature osteoclasts.[50] Furthermore, osteoclasts intercalated with BPs show morphological changes, such as a lack of the ruffled border[51] and disruption of the actin ring.[52] In addition, investigations of the molecular mechanism of the direct action of BPs on osteoclasts have shown that they are involved in inhibition of the mevalonate pathway.[53] It was reported that BPs reduce cell viability at concentrations in the range of 10^{-4} M.[54,55] In addition, evidence has accumulated, showing that the effects of BPs on osteoclasts are indirectly mediated via osteoblasts.[56] These indirect effects on osteoclasts are likely to alter the secretion from osteoblasts of factors, such as interleukin-6, that regulate the differentiation or activation of osteoclasts.[57,58] Furthermore, BPs have been shown to enhance the proliferation, differentiation, and bone-forming activity of osteoblasts directly.[59] Therefore, these studies suggest that BPs affect bone metabolism via both osteoclasts and osteoblasts.[8–10,60] In bone remodeling, osteoclasts and osteoblasts work closely together, also in a spatial sense, at defined remodeling sites. Their activities are coupled: A decrease in bone resorption due to BPs leads to a reduction in bone formation to a similar degree. Thus, it was long thought that BPs would only slow down bone repair.[61] However, in bone repair, osteoblasts can work independently. A reduction in osteoclast activity can, therefore, be expected to shift the balance between formation and resorption toward increased net bone formation. During bone repair, BPs have an anti-catabolic or a net anabolic effect.[24,61,62] The use of BPs delivery systems, given the cellular effects of BPs discussed in this section, opens new fields and attractive possibilities for BTE.

Bone Tissue Engineering

Multifunctional 3D scaffold: dual role as matrices and drug delivery systems

Under normal conditions, bone tissue has the capacity to regenerate itself. However, critical-size bone defects require a bone substitute that fills the defect and regenerates the damaged tissue. Even though biocompatible and resorbable 3D matrices (scaffolds) have shown capabilities to direct and promote host bone cells proliferation, the incorporation of bioactive molecules such as growth factors, cytokines and hormones, and/or therapeutic drugs can substantially improve the osteogenic potential of the scaffolds for the stimulation of bone regeneration and repair.[3,46,63,64] Therefore, the challenge for tissue engineers is to design and develop temporary bone scaffolds with suitable mechanical properties and the capability to be resorbed in concert with the ongoing bone formation, which should be also able to incorporate bioactive molecules and/or therapeutic drugs and to deliver them to the repairing site in a controlled manner; thus, extending the scaffold's biological functionality. A variety of materials have been employed in the elaboration of 3D scaffolds for BTE, most commonly bioactive and resorbable inorganics biomaterials, including calcium phosphates (CaP) (such as HA and tricalcium phosphate), mesoporous silica,[65] bioactive glasses,[46,66–72] and biodegradable polymers, both natural (e.g., collagen, chitosan, alginate) and synthetic, such as poly (D,L-lactide), poly(D,L-lactide-co-glycolide) (PLGA), and polylactide-co-glycolide copolymers.[73–75] Generally, the rationale of using polymers as drug delivery vehicles is based on the hydrolytically reactive unions in their backbone, which allow the tuning of their biodegradation rate; thus, encapsulated drugs can be released as the polymer degrades. Further, composites of inorganic biomaterials and polymers are being developed in order to improve the mechanical and biological properties of scaffolds, exploiting the flexibility of the polymers and the bioactivity of bioactive glasses or CaP.[69,76–92] It is expected that

multifunctional scaffolds will be able to maximize the access of bioactive molecules and/or therapeutic drugs to the surrounding tissues, and to control the release of drugs or growth factors in order to sustain the desired concentration level in their specific sites for the necessary period of time to stimulate bone formation.[93] In this context, the effect of drug incorporation on the physicochemical and mechanical properties of scaffolds and the capability of releasing drugs from scaffolds in predictable and reproducible kinetics are issues to be taken into account for the design of advanced functional scaffolds. In general, changes in the mechanical properties can be caused by some chemical interaction between the constituents of the scaffold and the loaded drug (e.g., chelation), which can also modify the kinetics of the dissolution reaction of the scaffold. On the other hand, it is well known that the release of drugs from a matrix depends on several factors, such as the type of union between drug and matrix as well as the microstructure of the matrix and its mechanism of degradation. Generally speaking, the release kinetic of drugs within 3D scaffolds for tissue engineering is linked to the degradation kinetic of the scaffold (a biodegradable matrix by definition). However, most multifunctional scaffolds studied as a matrix for drug release have been shown to have rates of degradation much lower than the required rate of drug release.[40,94–96] Thus, it is important to recognize that the drug release from a scaffold can be mainly controlled by the process of diffusion through it, at least during the first stages of the process. In addition, during the degradation of the scaffold, the dissolution of bioinorganic components is followed in most cases by the formation of an apatite surface layer and precipitation of HA, which will also affect the drug release kinetics. In the case of scaffolds elaborated from bioinorganic materials, it is possible to modulate the rate of scaffold degradation by varying the chemical composition, microstructure, and percentage of crystallinity.[34,35,40,66] Further, it is important to highlight that the development of these complex systems for the delivery of BPs should involve first the characterization of the drug release kinetics *in vitro.* Subsequently, *in vivo* studies should assess the effectiveness of the 3D scaffold that acts as a matrix for *in situ* administration and controlled release of loaded BP, the conservation of scaffold bioactivity, the BP distribution zone, and the occurrence of possible undesirable effects, for example, if migration of the drug out of the region of interest occurs. Finally, clinical assays should evaluate the performance of the system. An important issue to be taken into account is the way to quantify the release of BPs from those delivery systems. Due to the lack of chromophore groups, BPs are complex molecules that are analyzed by simple techniques of quantifications, and, in many cases, a sample pretreatment is required, most commonly, derivatization as a previous step for high-performance liquid chromatography quantification.[97] Different methods that quantify BPs in biological or pharmaceuticals samples were reported in the last years, and a summary of the most common ones is presented in Table 1.

BTE using BPs

Different strategies for the delivery and sustained release of BPs in the nearby host tissue have been investigated. Figure 2a summarizes the different strategies that incorporate BPs in scaffolds for BTE. BPs loading in CaP scaffolds was first reported to be via chemical or physical sorption on the CaP surface, which resulted in limited drug loading content and burst release on administration.[32,36,129–131] The use of these techniques may produce unspecified irregular shapes and sizes of the carriers and heterogeneous drug distribution within CaP, and, thus, the release kinetics would be without control. One of the strategies very often reported is based on the chelating capacity of BPs due to their two oxygen ions from bilateral phosphonate groups; most commonly, chelation between BPs and bioactive inorganic materials has been reported (Fig. 2a, items iii-iv). Although matrices for drug delivery are usually polymers, the bioactive inorganic materials that are usually employed in BTE scaffolds such as CaP and bioactive glasses with different solubilities and chemical compositions are attractive as drug carriers as well. Most commonly, the linkage of the drug to inorganic materials is made by soaking the inorganic scaffold in a BP solution, and recent examples of this method have been published in the last few years.[32,34,36,37,40,132–135] In a relevant study, Boanini *et al.* (2008)[132] synthesized HA nanocrystals loaded with different ALN contents (3.9, 6.2, 7.1 wt%). The *in vitro* evaluation showed that the presence of the BP in the composite nanocrystals produced a reduction in the number of osteoclasts of approximately 30% and an increase in osteoblastic growth and differentiation.[132] In particular, osteoblasts cultured at 6.2 wt% and 7.11 wt% content of ALN displayed an increase in the synthesis of alkaline phosphatase, osteocalcin, and type I collagen.[132] Moreover, it was shown that the dissolution rate of CaP can be modulated by modifying the percentage of crystallinity of the CaP phase, which is stable in water, and, thus, the dissolution rate is reasonably slow.[136] Thus, the modulation of the dissolution rate of CaP may enable control of the release rate of incorporated BPs.[136,137] A variety of this strategy includes the development of CaP-BPs coatings over polymer scaffolds (e.g., made of starch)[138] (Fig. 2a, item v*)*. In addition, the chelation capability between BPs and bioinorganic materials such as CaP and bioactive glasses has been utilized to prepare BP-loaded microspheres. For example, bioactive glasses were used by Välimäki *et al.* (2006)[133] to develop microspheres containing Zoledronate (ZOL), and CaPs were utilized for elaboration of BPs-loaded CaP microspheres.[32,33,36,40,129,130] Microspheres can be conveniently used for the elaboration of scaffolds for BTE (Fig. 2a, items ii and iii**)**. In a related investigation, ALN (25–45 mg) was loaded *in situ* during microsphere formation by a two-step process using water-in-oil microemulsion and subsequent HA nucleation/growth in water droplets.[40] ALN release was sustained *in vitro* over 40 days with linear release kinetics, except for an initial burst release during the first 24 h. An *in vitro* correlation was found between the controlled release kinetics of ALN and the dissolution rate of HA microspheres, indicating that the ALN release was induced by the HA mineral dissolution. In addition, *in vitro* studies showed the potential of HA-ALN microspheres on inhibiting osteoclastogenesis derived from monocytic macrophages. Other developments involve the entrapment of BPs in polymeric nano/microspheres that are loaded onto or within the surfaces of ceramics or composite scaffolds.[7,139] Shi *et al.* (2009)[7] have exploited the properties of both HA and PLGA by hybridizing them into composite microspheres in which ALN was loaded ("PLGA/HA-ALN"

Table 1. Methods for Bisphosphonates Quantification

Bisphosphonates	*Technique*	*Detection*	*LOD*	*LOQ*	*Sample*	*References*
Alendronate	HPLC-RP	Fluorimetric	-	-	urine	98
	HPLC-RP	Electrochemical	0.2 ng/mL	1 ng/mL	urine	99
	HPLC-RP	UV	-	-	pharmaceuticals	100
	HPLC-RP	Fluorimetric	-	1 ng/mL	human plasma	101
	HPLC-RP	Fluorimetric	-	3.5 ng/mL	urine	102
	HPLC-RP/MS	Mass spectrometry	4.86 μg/mL	6.667 ng/mL	urine	103
	HPLC-RP/MS^2	Mass spectrometry	0.05 ng/mL	-	urine	104
	ICP	ELSD	16 μg/mL	-	-	105
	ICP	UV	-	14 μg/mL	pharmaceuticals	106
	ICP	Fluorimetric	-	0.6 ng/mL	urine	106
	IC	Conductivity	-	0.005 mg/mL	pharmaceuticals	107
	IC	Refractive index	0.4 μg/mL	-	pharmaceuticals	108
	CZE	Conductivity	3.1 μM	9.3 μM	urine	109
	CZE	Indirect UV	50 μg/mL	-	standard	110
Pamidronate	HPLC-RP	Fluorimetric	0.1 μg/ml	-	urine	111
	HPLC-RP	Fluorimetric	0.5 μg/mL	-	blood	
	HPLC-RP	Fluorimetric	-	0.8 μmol/L	plasma	112
	HPLC-RP	Fluorimetric	-	0.7 μmol/L	urine	
	HPLC-RP	Fluorimetric	11.6 ng/mL	233 ng/mL	urine	113
	ICP	Fluorimetric	1 ng/mL	3 ng/mL	urine	114
	ICP	UV	0.1 μg/mL	0.1 μg/mL	pharmeceuticals	115
	ICP	Fluorimetric	-	-	urine	116
	ICP	ELSD	18 μg/mL	-	-	105
	ICP	Fluorimetric	10 ng/mL	20 ng/mL	serum	117
	IC	Conductivity	25 μg/mL	-	pharmaceuticals	118
	CZE	Conductivity	13.4 μM	39.3 μM	urine	109
	GC	Flame photometric	100 ng/mL	-	biological fluids	119
Ibandronate	ICP	ELSD	176 μg/mL	-	-	120
Zoledronate	ICP	ELSD	17 μg/mL	-	-	105
	ICP	UV	8 μg/mL	-	pharmaceuticals	121
Etidronate	ICP	ELSD	15 μg/mL	-	-	105
Clodronate	ICP	ELSD	37,5 μg/mL	-	-	122
	CZE	indirect UV	0.4 mg/ml	-	liposomal formulations	123
	CZE	indirect UV	30 μg/mL	-	standard	110
	GC	nitrogen-phosphorus detector	-	0.3 μg/mL	plasma	124
			-	0.5 μg/mL	urine	
Risedronate	IPC	UV	30 ng/mL	100 ng/mL	pharmaceuticals	125
	IPC	UV	0.48 μg/mL	1.61 μg/mL	pharmaceuticals	126
	IPC	UV	7 ng/mL	10 ng/mL	rat plasma	127
	IPC	UV	-	7.5 ng/mL	human urine	128

HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; RP, reverse phase; ICP, inductively coupled plasma; ELSD, evaporative light-scattering detector; CZE, capillary zone electrophoresis; GC, gas chromatography; PAM, photoacoustic microscopy; PCL, poly(ε-caprolactone).

system). The affinity of ALN to HA increases the encapsulation efficiency of the antiresorptive drug in the microsphere, particularly when ALN is preattached to HA particles before being in contact with the PLGA-containing organic phase, reaching more than 90% of ALN encapsulation. *In vitro* release of ALN over 30 days from PLGA/HA-ALN microspheres showed an exponential tendency despite a minimal initial bursting. After 30 days of incubation, 70%–90% of ALN was released from each sample. In addition, the *in vitro* tests carried out using human fetus osteoblast cultures showed that the release of ALN from PLGA/HA-ALN microspheres inhibited the growth of macrophages and enhanced the proliferation and activity of osteoblasts. Other strategies more recently employed use electro- and wet-spinning techniques for the production of polymeric fibrous scaffolds loaded with BPs and phosphate crystals (Fig. 2a, items ii).[140,141] Puppi *et al.* (2010)[140] developed, by electro- and wet-spinning, composite scaffolds made of poly(ε-caprolactone), HA nanoparticles (HANP), and CLO. The combination of electrospun and wet-spun fibres allowed the production of a multiscale network structure, and the binding affinity between ALN and HA was exploited by preparing a CLO-HANP complex before producing the fibres.[140] The inclusion of CLO-HANP complex particles into polymeric fibres further increased the osteoconductivity of the scaffold due to the presence of the inorganic phase. In addition, the physical binding between CLO and HA allowed further control over CLO release kinetics.[140] In a recent investigation, sandwich structure-like meshes by using the electrospun technique were developed by Lu *et al.* (2011).[141] Poly (*L*-lactic acid) (PLA) fibres were electrospun as a bottom layer of the structure; a solution of ZOL and polyethylene oxide constituted the middle layer; and, finally, other

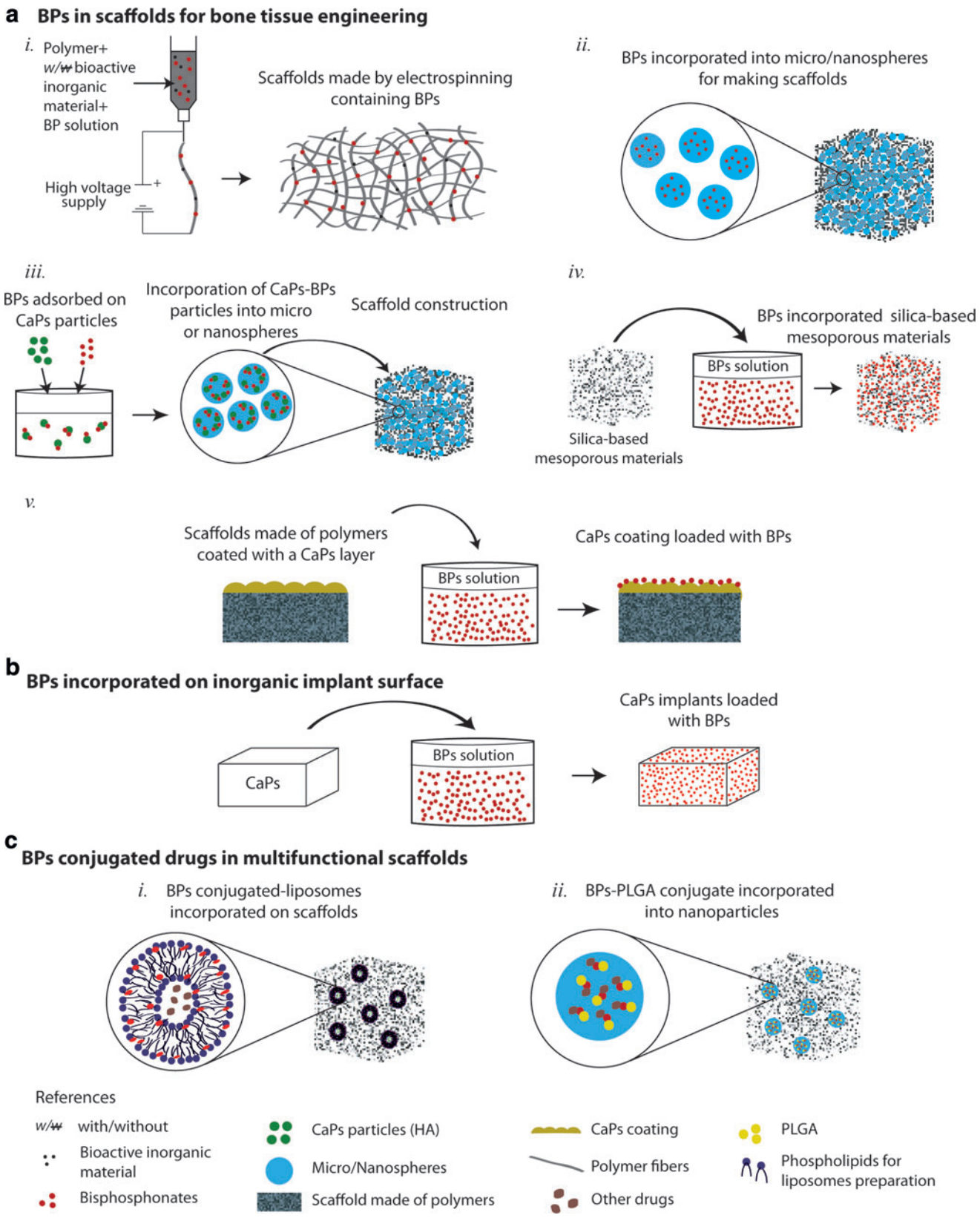

FIG. 2. Different strategies for incorporating BPs in scaffolds for bone tissue engineering **(a)** and **(c)** or implants **(b)**. Figure 2*ci* shows the utilization of BPs conjugates to incorporate other drugs into multifunctional scaffolds.[7, 32, 34,36,37,40, 134,135,138,139, 140,141,147,151,152] CaP, calcium phosphate; PLGA, poly(D,L-lactide-co-glycolide). Color images available online at www.liebertonline.com/teb

layers of PLA fibres were electrospun on the top.[141] *In vitro* release studies revealed that the initial burst effect and drug release kinetics can be controlled by adjusting the thickness of the electrospun barrier mesh and the drug-loaded mesh (e.g., the burst effect can be diminished by the increase in the thickness of the electrospun barrier mesh and the drug-loaded mesh).[141] Mesoporous silicas have been introduced to provide a dual role of scaffolds for bone tissue regeneration and matrices for the controlled delivery of BPs (Fig. 2a, items iv).[65] Silica-based mesoporous materials constitute a relatively new generation of materials that show ordered arrangements of channels and cavities of a different geometry built up from silicon dioxide (SiO_2) units.[72,142–144] The bioactive behavior of silica-based ordered mesoporous materials is undoubtedly an added value when considering these materials for bone tissue regeneration. The high pore volume of silica-based mesoporous materials enables the hosting of relative large amounts of biologically active molecules. In addition, their ordered pore network allows a fine control of the molecule load and release kinetics. In addition, the possibility of functionalizing the silanol-containing surface with different organic groups depending on the molecule to be adsorbed allows a better control over molecule loading and release.[65,134,135] The combination of bioactivity and controlled delivery capability is a remarkable synergy that has promoted increased research efforts on these materials for the manufacture of multifunctional 3D scaffolds for BTE in the last few years.[35,65,71,72,134] Of relevance for the present review, mesoporous materials such as SBA-15 were used by Balas *et al.* (2006)[134] and Nieto *et al.* (2008)[135] to design drug delivery systems for ALN. Amino groups were covalently grafted to the silanol groups on the pore surfaces of the mesoporous material to increase the bisphosphonate adsorption (up to 3-fold).[134,134] It was shown that a range of amine-functionalization degrees led to diverse adsorption rates of sodium ALN, and to different drug release profiles.[134,135] With high-surface and small mesoporous areas, the diffusion of bisphosphonate molecules to liquid media from mesoporous materials was observed as following a first-order kinetic model, which predicts a surface-dependent phenomenon.[145,146] The decrease in mesopore surface area due to the functionalization as well as the partial surface dissolution of the amino-modified silica matrix after long periods in relevant medium may induce slight changes in the BPs release mechanism.[134,135] Different diffusion processes or even a dissolution-diffusion process can be interpreted by using a zero-order or lineal model.[134,135] Table 2 summarizes several published studies on multifunctional scaffolds loaded with BPs. The analysis of the literature confirms that even though it is clear that the concept of incorporating a BP in multifunctional 3D scaffolds for BTE is in its early years, the research carried out in the past decade has put forward several approaches highlighting its great potential. In this sense, it is expected that time will elapse before clinical results of relevance are achieved. A large body of biological information is still needed to fully understand the performance both *in vitro* and *in vivo* of the BPs incorporated in 3D scaffolds for BTE. Once obtained, this information will be advantageous to provide a rational design of the scaffold and the optimization of its function as a controller of the release of BPs. In an ideal situation, the release of BPs should be finely tuned to match the physiological needs of bone as it regenerates. In this sense, little is known yet on the specific amount of BP that is needed to be released from the scaffold in relation to a particular local microenvironment, the clearance of the drug from the zone, and how the new vascularization induced by the presence of the scaffold would affect the residence time of the drug.[3,173] In addition, there is still considerable research and development work to be done in terms of generating a deep understanding on how the BPs release kinetics is affected by the variable resorption rate of the matrix and by the morphological changes caused during scaffold degradation *in vitro* and *in vivo*, in particular considering bioactive inorganic scaffolds in which an HA layer forms onto its surface *in situ*. It is also important to highlight that due to the lack of any standard technique available to perform *in vitro* release studies which evaluate the suitability of the different bone scaffolds proposed as BPs delivery systems, it is very difficult to compare the different results obtained by different research groups. Variables such as composition and flow speed of sampling media, sampling technique, period of study, and drug stability are different for each study, making comparison a very difficult task.

Bisphosphonate-conjugated drugs in multifunctional 3D scaffolds

The strong interactions between BPs and the inorganic components of bone has led to the exploration of the benefit of associating BPs to therapeutic drugs, including radioisotopes (such as samarium-153 complexed to tetraphosphonate), anti-inflammatory and anti-neoplastic drugs, as well as bioactive molecules such as cytokines and growth factors by conjugation through a specific linkage (Fig. 2c, items *i* and *ii*),[148] thus acting as an osteotropic drug delivery system.[149,150] In this context, Pignatello *et al.* (2009)[151] proposed a nanocarrier developed from the PLGA-ALN conjugate. ALN was bound covalently to a free-end carboxylic group of PLGA. In a second step, nanoparticles with a mean size of 200–300 nm were obtained by a classical solvent-evaporation method. The authors demonstrated *in vitro* that nanoparticles do not affect osteoblast and endothelial cell viability. In addition, it was shown that PLGA–ALE nanoparticles were adsorbed onto HA to a higher extent than pure PLGA nanoparticles due to the presence of ALN. Even though the prepared conjugate represents a novel biomaterial, no drug was loaded into the system. A step further was proposed by Wang *et al.* (2011)[152] through the development of a drug delivery system by combining composite scaffolds made up of collagen and HA (Col/HA) with bisphosphonate (2-(3-mercaptopropylsulfanyl)-wthyl-1, 1-bisphosphonic acid)-derivatized liposomes. In this case, the affinity between HA and BPs was useful for the controlled release of the drugs from BP-liposomes in Col/HA scaffolds. The Col/HA material was prepared by a freeze-drying method that yields a porous scaffold. Three different model drugs, carboxyfluorescein, doxorubicin, and lysozyme, were entrapped in liposomes; no difference was observed in drug release between BP-derivatized liposomes and free liposomes *in vitro*.[152] Unencapsulated drugs and drugs encapsulated in non-derivatized liposomes were seen as displaying rapid release from the scaffolds, whereas the drugs entrapped in BP liposomes showed a slower release from the Col/HA scaffolds due to the BP high-binding affinity for calcium ions in the HA structure, which enhances the adsorption of BP-liposomes onto Col/HA scaffolds.[152] Wang *et al.* 2011 provided a

Table 2. Systems Containing Bisphosphonates That Can be Used to Develop Scaffolds for Bone Tissue Engineering

Delivery systems that can be used as scaffolds	*BP incorporate*	*Experimental trial*	*BP release quantification*	*Most important contributions*	*References*
Chitosan microspheres	Pamidronate	*In vitro, In vivo*	-	The release of BP from microspheres was faster *in vitro* than *in vivo*. After implantation, drugs exhibited a relatively increased disposition in the adjacent tibia.	139
Poly-D,L-lactic acid (PDLLA) scaffolds	Pamidronate	*In vivo*	-	PDLLA pellets containing Bone Morphogenetic Protein (BMP) and PAM showed an increase in bone formation after 3 weeks when low doses of PAM were used (0.02 mg). Polymer degradation remained until 8 weeks.	147
Mesoporous silica-based materials (MCM41, SBA15)	Alendronate	*In vitro* release of Alendronate	-	Amine functionalization on mesopores enhances 3 times the incorporation of ALN and reduces the mesopore surface area. The diffusive behavior of the absorbed molecule through the mesopores can be calculated using a zero-order or lineal model.	134
Mesoporous silica-based materials (SBA15)	Alendronate	*In vitro* release of Alendronate	RP-HPLC	Mesopores functionalizatiwith aminopropyl groups was made using a catalytic or an anhydrous procedure. The catalytic method induces a more gradual ALN loading (approximately 3 mg ALN/25 mg SiO_2) that depends on the functionalization degree and offers a better control in the release of ALN molecules showing a deviation from the theoretical first-order behavior.	135
HA-coated starch scaffold	Clodronate	*In vitro*	HPLC-UV detection	The microhardness increases by increasing the BP concentration on the coating (from 0.004 to 1 mg/cm^2). A zero-order kinetic was observed during 14 days at pH 7.4. The CLO that is incorporated promoted osteoblast-like cell adhesion, and it has influenced cellular proliferation in a way dependent of the concentration, being 0.02 mg/cm^2 of BP the ideal concentration for enhancing cell viability.	138

(*continued*)

Table 2. (Continued)

Delivery systems that can be used as scaffolds	*BP incorporate*	*Experimental trial*	*BP release quantification*	*Most important contributions*	*References*
PLGA/HA microspheric system	Alendronate	*In vitro*	Spectrophotometrically, as a Fe(III) complex	ALN was encapsulated using a single emulsion method, which showed a higher encapsulation efficiency (about 90%) than the double emulsion one. A controlled zero-order release during 30 days was achieved, without a remarkable initial burst effect: composites with 50% of HA showed a better controlled release. Inhibition on the growth of macrophages and enhancement in the proliferation of osteoblasts were observed.	7
PCL fibres loaded with HA and BP	Clodronate	*In vitro* characterization	-	PCL fiber scaffolds were developed by using electro- and wet-spinning techniques and loaded with HA nanoparticles, which had CLO linked (about 75 mg ALN/250 mg HA). Release kinetics of CLO, through the tuning of fiber dimensions and mesh porosity, could be controlled.	140
PLA/PEO-ZOL/PLA nanofiber meshes	Zoledronate	*In vitro* release of Zoledronate	HPLC-UV detection	The sandwich structure-like meshes show the main advantages of facile preparation condition and the possibility of including hydrophobic or hydrophilic drugs. *In vitro* experiments revealed that with an increase in the thickness of inner drug-loaded mesh, the drug release rate and initial burst release decreased.	141
HA microspheric system	Alendronate	*In vitro*	Spectrophotometrically, as a Fe(III) complex	They fabricated a microsphere-type carrier where ALN loading and microsphere formation can occur through a simultaneous process, and the loading content was much higher than other CaP carrier systems (approximately 19.5 wt.% ALN). A controlled drug release for 40 days was achieved, which was dependent on the dissolution rate of the HA microspheres. The release showed linear kinetics, except for a burst effect during the initial 24 h. In addition, the inhibition of osteoclast formation was observed.	40

BP, bisphosphonates; RP, reverse phase; ALN, alendronate; HA, hydroxyapatite; PCL, poly(ε-caprolactone); CLO, clodronate; PLA, Poly (*L*-lactic acid); PEO, polyethylene oxide; ZOL, Zoledronate; CaP, calcium phosphates; UV, ultraviolet.

potential drug release platform that is used in bone regeneration and repair.[152] However, it would be important to determine whether the rate of HA dissolution could modify the rate of liposome release *in vivo*.

Role of BPs as coatings for improved fixation of orthopedic implants

Although not a "classical" tissue-engineering approach, relevant research on the use of BPs to improve the fixation of orthopedic implants is discussed in this section for completeness and for the relevance of this review to the topic. The fixation of orthopedic implants in bone relies strongly on the initial stability of the implant. When the initial stability is not achieved, then micromotions occur at the bone implant interface.[153,154] Micromotions activate premature bone resorption and remodeling by osteoclasts,[155] which result in periprosthetic osteolysis and later implant migration and wear, limiting the implant longevity.[156] BPs incorporated into orthopedic implants can be used to reduce periprosthetic osteolysis at the implant/bone interface, allowing orthopedic implants to achieve a stronger primary fixation[157] by the inhibition of osteoclast action.[158] The materials that are most widely used to fabricate implants for bone therapy are Titanium (Ti) and Ti alloys.[60,131,158–164] Several strategies have been developed to incorporate BPs onto the surface of titanium implants (Fig. 3). Surface modifications with CaPs were reported as being very attractive, because BPs have a marked affinity to these substances as explained in previous sections.[159] CaPs, most commonly HA, have been used to generate thin coatings on the surface of titanium implants.[60,131,159,158,160,161,165] The most common strategy that produces the chemical association of BPs with the CaPs coating is by soaking the implants in a BPs solution (Fig. 3) [60,131,158–164] Other strategies that produce the chemical association of BPs with CaPs reported include bisphosphonate adsorption onto plasma-sprayed HA and simulated body fluid-grown HA coatings that are commonly used for orthopedic implants.[131,158,164] Table 3 summarizes different methods for titanium surface modification that have been used for BPs fixation.

Conclusions

Research carried out in the past decade has put in evidence the great potential of incorporating BPs in multifunctional scaffolds for bone regeneration applications. Especially encouraging are the results obtained with composite 3D scaffolds for BTE. The technologies and strategies summarized and discussed here represent significant progress achieved toward the development of multifunctional scaffolds with the added value of the controlled release of BPs at the desire site, thus enhancing the scaffold potential performance *in vivo*. However, and despite several obstacles already overcome by the research, there is still a significant gap that should be bridged to meet the complex requirements for effective bone regeneration *in vivo*. Among the issues to be addressed, it is important to highlight the difficulties of achieving controlled release rates, avoiding initial burst release, and the important lessons to be learned from the need to improve drug loading on scaffolds, especially when the matrix is made of bioactive inorganic materials. In this context, it is essential to know how the BPs release kinetics is affected by the variable resorption rate of the scaffold and by the morphological changes caused during its degradation *in vitro* and *in vivo*, in particular, considering the *in-situ*

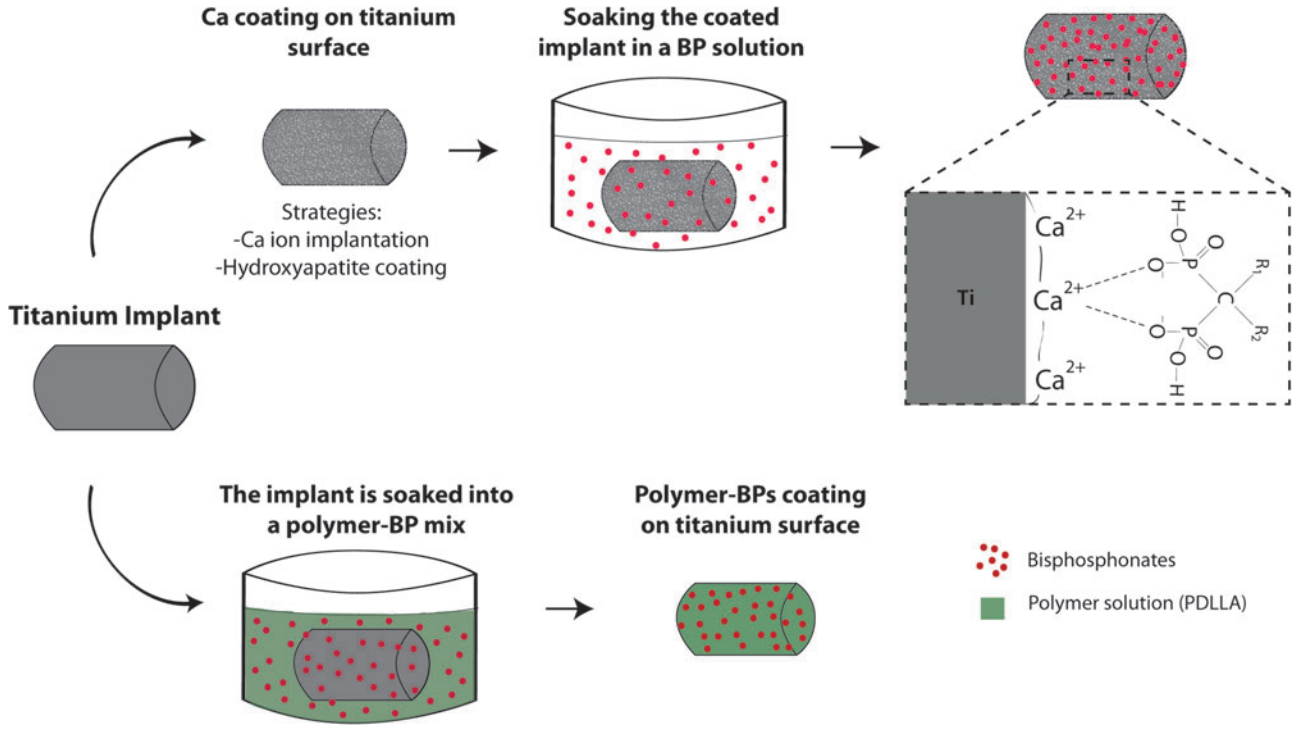

FIG. 3. Different strategies employed for binding BPs to coated titanium.[60,131,158–164] Color images available online at www.liebertonline.com/teb

Table 3. Different Methods for Surface Titanium Modification That were Used for Bisphosphonates Fixation

Bisphosphonate	*Method for BP immobilization on titanium surface*	*Qualitative/Quantitative analysis for BPs fixation*	*Experimental trial*	*BP release quantification*	*Most important contributions*	*References*
Pamidronate	Ca ion-implanted titanium and hydroxyapatite coating	X-ray photoelectron spectroscopy (qualitative)	*In vitro*	-	They achieved incorporating the Pamidronate into the Ca-coated implant: it was not toxic for osteoblastic cells and for inhibiting the adherence of *P. gingivalis,* which is important for dental applications.	159
	Ca ion-implanted titanium	X-ray photoelectron spectroscopy (qualitative)	*In vivo*	-	The incorporation of BP into the surface of a Ca-coated Ti implant accelerates new bone formation around the implant in comparison with Ti and Ca-Ti implants	60
	Hydroxyapatite coating on titanium	-	*In vivo*	-	The molecular precursor used for implant coating allows that Ca coating of any shape can be deposited. The incorporation of BP into the implant showed a better bone formation than untreated Ti and apatite-Ti.	160
	Co-precipitation directly onto the Ti surface and Hydroxyapatite coating	X-ray photoelectron spectroscopy (qualitative)	*In vitro*	Alternating ionic current (AIC) conductivity measurements, during 24 h.	For BP incorporation into the implant, co-precipitation and fast loading methods were used, showing the simplicity of both methods. The Pamidrotante present on the surface of the fast-loaded HA coatings was strongly bound, which makes a slower release.	161
Zoledronate	Hydroxyapatite coating on titanium	Determination of the phosphorous content: Ames method (quantitative)	*In vivo*	-	The local delivery of BP incorporated on calcium phosphate-coated Ti allowed for an increase in the mechanical fixation of an orthopedic implant. Bone volume fraction is dependent on the ZOL content of the coating: implants (volume: 35.3 mm^3) containing 8.5 μg ZOL induced the highest mechanical stability.	131
	Poly(D,L-lactide)-BP coating	-	*In vitro*	-	ZOL incorporated (from 10 to 50 μM) in a poly(D,L-lactide) coating of a Ti implant inhibited osteoclast formation and reduced their resorption activity.	162

(*continued*)

Table 3. (Continued)

Bisphosphonate	*Method for BP immobilization on titanium surface*	*Qualitative/Quantitative analysis for BPs fixation*	*Experimental trial*	*BP release quantification*	*Most important contributions*	*References*
	Hydroxyapatite coating on titanium	Determination of the phosphorous content: Ames method, ^{31}P NMR (quantitative)	*In vivo*	-	The incorporation of ZOL (2.1 μg ZOL/ 35.3 mm^3 implant) on HA-coated implants improved its fixation, which was confirmed by the increase of periprosthetic bone density.	158
	Hydroxyapatite coating on titanium	-	*In vivo*	*In vitro* release of ZOL in buffer media during 21 days, quantified by HPLC.	HA-coated Ti implants were loaded with ZOL and bFGF. The *in vitro* release test showed that the amounts of ZOL and bFGF released from implants treated with ZOL + bFGF were low during the first days. The maximal amount of new bone ingrowth into HA-coated implants was found in the rats treated with both agents. The use of ZOL and bFGF effectively increased trabecular microarchitecture parameters to a greater degree than the use of ZOL or bFGF alone.	163
Zoledronate Pamidronate Ibandronate	Hydroxyapatite coating on titanium	-	*In vivo*	*In vitro* release of BPs for 21 days, quantified by HPLC using an UV detector.	Three different HA-coated Ti implants were made (ZOL-implant; PAM-implant; Ibandronate-implant). Immobilized BPs had positive effects on implant fixation in osteoporotic bone, promoting peri-implant bone formation and improving its mechanical properties. The release rate of BPs in the first few days was slightly different, but three BPs were still detectable during 21 days, which was a key period for the early bone formation and, thus, more important for peri-implant bone formation and implant-bone osseointegration. The three BPs used in this study have different levels of efficacy, with a rank order of ZOL > Ibandronate > PAM.	164

formation of an HA layer on the surface of bioactive inorganic scaffolds. Other issues include the need to be certain about the specific amount of BP that is needed to be released from the system, also taking into account the physiological constant clearance of the drug from the microenvironment in *in vivo* situations and also the effect of new vascularization induced by the presence of the scaffold. In addition, for successful clinical and commercial development, considerations should also be given to the ability to cost effectively manufacture the delivery system and to obtain approval from the appropriate regulatory authorities. These last two important requirements are often overlooked.

Acknowledgment

This work was supported by ANCyT (grant PICT 0138).

Disclosure Statement

No competing financial interests exist.

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Received: December 25, 2011
Accepted: March 20, 2012
Online Publication Date: May 11, 2012