Adsorption of benzoxaboroles on hydroxyapatite phases

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Abstract

Benzoxaboroles are a family of molecules that are finding an increasing number of applications in the biomedical field, particularly as a "privileged scaffold" for the design of new drugs. Here, for the first time, we determine the interaction of these molecules with hydroxyapatites, in view of establishing (i) how benzoxaborole drugs may adsorb onto biological apatites, as this could impact on their bioavailability, and (ii) how apatite-based materials can be used for their formulation. Studies on the adsorption of the benzoxaborole motif ($C_7H_7BO_2$, referred to as BBzx) on two different apatite phases were thus performed, using a ceramic hydroxyapatite (HA_{ceram}) and a nanocrystalline hydroxyapatite (HA_{nano}), the latter having a structure and composition more similar to the one found in bone mineral. In both cases, the grafting kinetics and mechanism were studied, and demonstration of the surface attachment of the benzoxaborole under the form of a tetrahedral benzoxaborolate anion was established using ^{11}B solid state NMR (including ^{11}B-^{31}P correlation experiments). Irrespective of the apatite used, the grafting density of the benzoxaborolates was found to be low, and more generally, these anions demonstrated a poor affinity for apatite surfaces, notably in comparison with other anions commonly found in biological media, such as carboxylates and (organo)phosphates. The study was then extended to the adsorption of a molecule with antimicrobial and antifungal properties (3-piperazine-bis(benzoxaborole)), showing, on a more general perspective, how hydroxyapatites can be used for the development of novel formulations of benzoxaborole drugs.
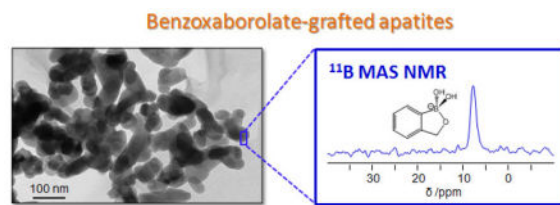
Graphical Abstract

Benzoxaborolate-grafted apatites

11B MAS NMR

Keywords

Hydroxyapatite; benzoxaborole; surface modification; surface characterization

1. Introduction

The unique reactivity of the benzoxaborole group [1–3] as well as its lack of toxicity [3–5] have recently attracted much attention for the development of new drugs (Figure 1). Indeed, over the past ten years, a series of therapeutic molecules containing the benzoxaborole function have been synthesized, with antibacterial, antiviral, anticancer, anti-parasitic or anti-inflammatory activities [1,6]. Among these, tavaborole is worth mentioning (AN2690 – Figure 1b), as it was recently commercialized by Anacor Pharmaceuticals for the topical treatment of onychomycosis [1,6]. Another important molecule under investigation is AN5568 (also referred to as SCYX-7158 – Figure 1c), an oral drug candidate against human African trypanosomiasis (sleeping sickness) [1,6]. The ability of the benzoxaborole group to bind to diols at physiological pH has also been exploited for other biomedical applications, like the internalization of enzymes in cells [7], the separation of sugars [8], or the rapid enrichment of glycosylated proteins [9].

While many studies have looked into the reactivity and biological activity of benzoxaboroles in solution [1,10], little is known as to how these molecules interact or react with common biomaterials, such as those used for the formulation of drugs or the elaboration of implants. It is only recently that our group started to study the association of simple benzoxaboroles (including AN2690) with inorganic biomaterials like layered double hydroxides (LDH) and with organic polymers like poly-L-lactic acid (PLLA) [11,12]. In both cases, the benzoxaboroles were incorporated into the biomaterials, their local environment was analyzed using techniques like solid state NMR, and their release in physiological media was studied. We found that performing such investigations from a fundamental perspective was necessary because it allowed evaluating the intrinsic reactivity and stability of the benzoxaborole function with respect to these biomaterials, and drawing conclusions which are of importance for the development of benzoxaborole drug formulations.

One unexplored aspect is the reactivity of benzoxaboroles with respect to natural biominerals like hydroxyapatite. These studies are necessary not only because hydroxyapatites, and more generally calcium phosphates, can be used for the formulation of drugs [13], but also because once introduced in the body, benzoxaboroles may encounter calcium phosphate crystallites (such as those found in bone). Indeed, bone is a tissue in which some drugs, administered for example through oral pathways, can accumulate by adsorption onto bone mineral [14]. This is the case for bisphosphonates like alendronate and

tiludronate [15], but also for tetracycline antibiotics like chlortetracycline and doxycycline [16]. Such an accumulation has an impact on the drug release kinetics. In a similar way, in the case of benzoxaboroles, the interaction of the molecules with the crystal surfaces of hydroxyapatites could influence their distribution and determine the drug efficacy. Thus the purpose of this study is to investigate, for the first time, the strength and mode of interaction of benzoxaboroles with hydroxyapatite surfaces.

Below, we will first describe the grafting of benzoxaboroles on ceramic and biomimetic hydroxyapatite phases, discussing in each case, the grafting kinetics, density and mechanism. Using the simplest benzoxaborole (BBzx, Figure 1a), we determine how this organoboron molecule reacts with calcium phosphate surfaces. Next, in order to understand the potential consequences of benzoxaborole/apatite interactions in a therapeutic context, we will compare the strength and mode of grafting of benzoxaboroles onto hydroxyapatite with other oxo-anionic molecules that are already present in the body and/or more commonly used to functionalize apatite surfaces, like carboxylates and organo-phosphates. Indeed, carboxylates are widely present in proteins like osteocalcin, which is proposed to bind to bone mineral through peripheral γ-carboxyglutamate moieties [17,18], as well as in small molecules like citrate, whose presence at the surface of bone mineral has been widely investigated in recent years [19]. Organophosphate groups are also exposed at the surface of post-translationally modified proteins like osteopontin, another bone protein known to interact strongly with hydroxyapatite [20]. Finally, we will show that more complex benzoxaborole molecules can also be grafted onto hydroxyapatite, such as the 3-piperazine-bis(benzoxaborole) molecule (bis-pipe-BBzx, Figure 1d), that displays a high biological activity against bacteria like *Mycobacterium luteum* and fungi like *Candida tenuis* and *Aspergillus niger* [10,21].

2. Materials and Methods

2.1 Materials

The 2-hydroxymethylphenylboronic acid cyclic monoester ($C_7H_7BO_2$, benzoxaborole, abbreviated BBzx, Figure 1a) was purchased from Sigma-Aldrich (97% purity), while the 3-piperazine-bis(benzoxaborole) molecule (abbreviated bis-pipe-BBzx, Figure 1c) was synthesized according to previously published procedures [22]. Sodium phenylphosphate ($C_6H_5PO_4Na_2$, ABCR, 98%, Karlsruhe - Germany) and benzoic acid (C_6H_5COOH, Acros organics, 99.5%, Geel - Belgium) were used as received.

Ceramic hydroxyapatite was purchased from BioRad (CHT Type II 40 μm, BioRad, Hercules, CA, USA). This phase is referred to as HA_{ceram}. SEM and TEM images show that the powder consists of beads of 20 to 50 μm diameter, each bead being composed of smaller crystallites of hydroxyapatite (see Figure S1 in supporting information). Prior to grafting experiments, 30 g of the ceramic HA were suspended in 150 mL of H_2O for 24 hours, and then dried at 100°C for 16 hours in vacuum, in order to homogenize the surface properties of the material prior to the grafting. The sample was stored at room temperature. The Brunauer Emmett Teller (BET) specific surface area of this powder, derived from N_2 physisorption at 77 K, was 22 m^2g^{-1}. XRD, ^{31}P and ^1H solid state NMR confirmed the presence of crystalline HA only.

The biomimetic hydroxyapatite phase, referred to as HA_{nano}, was synthesized following the procedure described by Pascaud *et al* [23]. In brief, it was prepared by introducing 100 mL of a calcium nitrate solution ($Ca(NO_3)_2, 4H_2O$, $[Ca^{2+}] = 0.295$ mol.L^{-1}) into 200 mL of an ammonium hydrogenphosphate solution (($NH_4)_2HPO_4$, '[P]' = 0.606 mol.L^{-1}). The excess of phosphate ions allowed the buffering of the solution. The suspension was stirred at room temperature for 5 minutes, and then transferred into a sealed container to mature at 37°C for 1 day (in absence of stirring). The precipitate was then vacuum filtered, washed 3 times with 100 mL of deionized water, and freeze-dried. Then the powder was sieved (<125 μm) and stored in a freezer. SEM and TEM images show that the powder consists of agglomerated nanocrystals. XRD shows the lower crystallinity of this phase, in comparison to HA_{ceram} (see Figure S1). The Brunauer Emmett Teller (BET) specific surface area of this powder, derived from N_2 physisorption at 77 K, was 162 m^{2}g^{-1}. According to elemental analyses, the Ca/P atomic ratio in HA_{nano} is 1.49 (with %$_{wt}$ (Ca) = 34.19 and %$_{wt}$ (P) = 17.76). It is worth noting that all these characterizations are consistent with those reported previously for biomimetic nanocrystalline apatites, in which the crystallites are formed of an apatitic core covered by an amorphous hydrated layer, which is prone to ionic exchanges [23,24].

Reagent grade solvents and ultrapure water were used in all reactions, and the pH of the solutions was adjusted by addition of a few drops of NaOH or HCl aqueous solutions (using solutions of 0.03, 0.3 and/or 3 mol.L^{-1}). PBS (phosphate buffer saline) was prepared according to established procedures (pH = 7.4, composition: NaCl ~ 137 mM, Na_2HPO_4 ~ 15 mM and KH_2PO_4 ~ 1.4 mM).

2.2 Grafting procedures and synthesis of crystalline model compounds

Different grafting procedures were used for the HA_{ceram} and HA_{nano} phases, two apatites being very different in terms of surface chemistry and reactivity.

For HA_{ceram}, grafting solutions were prepared in water, by dissolving the molecule of interest at concentrations ranging between 3 and 30 mmol.L^{-1} (depending on the molecule and the type of test performed), and adjusting the pH to 7.4 (using aqueous solutions of NaOH and/or HCl). In a centrifuge tube, 5 mL of grafting solution was then added, followed by 250 mg of pre-treated HA_{ceram}. The suspension was agitated at 22 °C by rocking at 50 cycles per minute for time periods ranging from 10 min to 1 week (Stuart SSL4 see-saw rocker, Bibby Scientific, Stone, UK). The suspension was centrifuged at 20000 rpm for 5 minutes. The supernatant was removed, filtered over 0.45 μm Millipore filters, and then stored in a refrigerator until further analyses (see below). The remaining powder was resuspended in 2.5 mL of ethanol, and the new suspension was stirred at room temperature for 10 minutes. The ethanol supernatant was removed after centrifugation, and this washing procedure was repeated once. The purpose of the ethanol washings was to remove the most weakly physisorbed species from the surface. The resulting powder was then dried under vacuum at 100 °C for ~16 hours. On average, ~200 mg of powder was recovered. Each type of grafting was performed between 2 and 5 times, depending on the parameter studied. Control samples were also prepared by suspending under the same conditions HA_{ceram} in an aqueous solution (pH 7.4) not containing any benzoxaborol(at)e, and then performing the same centrifugation, washing and drying steps as described above.

The grafting onto HA_{nano} was performed by adapting a protocol reported in the literature for the functionalization of biomimetic apatites by phosphonates [23]. In a centrifuge tube, 5 mL of an aqueous solution of BBzx were introduced (C ~16 to 80 mmol.L^{-1}; pH 7.4 aqueous solution, containing additional KCl at 1 mM), followed by 150 mg of HA_{nano}. The tube was closed, sonicated for 1 minute, and then incubated at 37°C for 6 h. The suspension was then centrifuged at 20000 rpm for 5 minutes. The supernatant was removed, filtered over 0.45 μm Millipore filters, and then stored in a refrigerator until further analyses (see below). The remaining powder was washed twice by dispersion in 2.5 mL of water, and the suspension was stirred at room temperature for 10 minutes. The powder obtained after these washings was freeze dried and then stored in a freezer. On average, ~110 mg of powder was recovered. Each type of grafting was performed between 2 and 5 times, depending on the parameter studied. Control samples were also prepared for these experiments.

To assist in the interpretation of the spectroscopic data, a model crystalline phase was synthesized, by precipitation of benzoxaborolate anions of BBzx by Ca^{2+}. This model material is referred to as $CaBBzx.2H_2O$, and details on its synthesis and characterization by powder XRD, IR and ^{11}B solid state NMR are provided in the supporting information (Figure S2).

2.3 Characterization

X-ray diffraction powder patterns were recorded using a PANalytical X'pert MPD-Pro diffractometer at the wavelength of Cu Kα1 (λ = 1.5405 Å) (45 kV and 20 mA) in Bragg-Brentano scanning mode. The program scanned angles (2θ) from 4 to 70° with a 0.017° step, and a step time of 40 s.

BET specific surface areas were measured by physisorption of N_2 using a Tristar instrument (Micromeritics, Norcross, GA, USA). Prior to measurements, samples were degassed under vacuum overnight at 100 °C for HA_{ceram}-derived phases, and at room temperature for HA_{nano} derived samples (in order to avoid the destruction of the amorphous hydrated surface layer and subsequent agglomeration of the crystallites).

SEM analyses were conducted on a Hitachi S4800 instrument under an excitation voltage between 0.5 and 8.0 kV depending on each powder's surface charging. Powdered samples were simply deposited on double sided tape and then Pt-metalized by sputtering under vacuum. TEM analyses were carried out at 100 kV using a JEOL 1200 EXII microscope. Samples were prepared by dispersing 2 mg of powder in 2 mL of a 1/1 $EtOH/H_2O$ mixture, sonicating the suspension for 10 minutes, and then depositing one drop of it onto a TEM Cu grid.

Solid state NMR characterizations were performed under MAS (magic angle spinning) on a Varian VNMRS 600 MHz NMR spectrometer (14.1 T), using a 3.2 mm T3 HXY Varian triple resonance probe. Experiments were performed spinning at 20 kHz and under temperature regulation, in order to ensure that the sample remains at room temperature inside the rotor during the analysis. ^{11}B MAS NMR experiments were performed at a frequency of 192.44 MHz. A DFS (double-frequency sweep) [25] enhancement scheme was applied (convergence sweep from 200 to 70 kHz; 5.0 ms pulse) prior to the excitation pulse

(1.25 μs, corresponding to a 45° solid pulse), and spinal-64 ^{1}H decoupling was applied during acquisition (100 kHz RF) [26]. The recycle delay was set to 1 s, and a total of ~2000 transients were acquired. ^{11}B-^{31}P correlation experiments were performed using a REDOR-type sequence (Rotational-Echo DOuble Resonance) [27]: a DFS excitation scheme was first applied on the ^{11}B side (using the characteristics mentioned previously), followed by an echo (using ^{11}B π/2 and π pulses of 2.5 and 5.0 μs, respectively, these pulse lengths being optimized directly on the sample). Rotor-synchronized echo delays ranging between 1.5 and 5.0 ms were used, and ^{31}P recoupling pulses of 10 μs were applied during these delays. Spinal-64 ^{1}H decoupling was applied during both the evolution and acquisition periods. The recycle delay was set to 1 s, and the total number of transients acquired ranged from 20000 to 22000, depending on the sample. An explanation to this ^{11}B{^{31}P} DFS-REDOR NMR experiment is provided in supporting information (Figure S3).

The amount of grafted molecules at the surface was determined by UV-vis spectroscopy. For this purpose, the samples were first "degrafted" by dispersing 50 mg of functionalized hydroxyapatite powder in 2 mL of PBS and stirred for 10 minutes at room temperature. The suspension was then centrifuged and the supernatant was recovered and filtered to remove any residual particles in suspension. The recovered "degrafted" powders were further analyzed, showing that any residual molecules potentially present at the surface of the material are beneath the limits of detection: for example, even after an overnight acquisition in ^{11}B solid state NMR, no signal due to residual boron could be detected in the material, thereby confirming that the desorption conditions were efficient in removing the grafted molecules in view of the titrations. The titration of the supernatant solutions was performed by UV-vis spectroscopy on a Perkin Elmer precisely Lambda 35 spectrometer, using the standard additions method [28]. For each grafted sample, the desorption of the benzoxaboroles and UV titration were performed in triplicate. The total calcium and phosphate content present in the supernatant at the end of the grafting was also analyzed using ICP-OES (Inductively-Coupled Plasma Optical Emission Spectrometry), on a Perkin Elmer "Optima 7000 DV" apparatus. For this purpose, the supernatant solutions recovered were diluted appropriately so that their "Ca" (or "P") concentration fell in the range of the calibration curve of the element, prior to titration.

3. Results

3.1 Kinetics, density and mechanism of grafting of the benzoxaborole function on hydroxyapatites

3.1.1 Grafting onto HA$_{ceram}$—The functionalization of HA$_{ceram}$ by BBzx was performed by suspending the apatite phase in a solution of BBzx at pH 7.4. Initial grafting tests were carried out at 22°C for 6 h, using a BBzx solution in which the benzoxaborole content exceeded by a factor ~2 the quantity needed to cover the HA$_{ceram}$ surface by a dense monolayer [29].[1] These conditions were chosen in order to ensure the presence of a sufficient amount of organoboron molecule at the surface of the material for the first

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[1]By analogy with previous studies on the grafting of small molecules like phenylphosphonic acid on inorganic surfaces [29], and supposing that the BBzx molecules are attached perpendicularly to the surface forming a monolayer, it was assumed that the maximum grafting density is ~5 molecules/nm^2.

characterizations. Powder X-ray diffraction analyses of this material showed that HA_{ceram} was the only crystalline phase present after the grafting protocol (Figure 2a). In particular, no secondary Ca-benzoxaborolate crystalline phases formed by dissolution-reprecipitation, as shown by the absence of diffraction peaks at low angles on the XRD powder pattern of the grafted HA_{ceram} phase. This was important to verify because previous studies on the grafting of organo-anions like phosphonates onto hydroxyapatite had demonstrated that such phenomena could occur, leading to the formation of crystalline calcium phosphonate by-products [30]. In the grafted HA_{ceram} material, the presence of organoboron species was shown using ^{11}B solid state NMR (Figure 2b): a resonance due to tetrahedral benzoxaborolate anions was identified [11]. Additional $^{11}B\{^{31}P\}$ REDOR NMR experiments, performed on the grafted HA_{ceram} phase (Figure 2c), revealed the proximity between the benzoxaborolates (^{11}B) and phosphates (^{31}P), suggesting that the benzoxaborolate anions are indeed located at the hydroxyapatite surface.

After demonstrating that it is possible to graft benzoxaborolates on HA_{ceram} in water at pH 7.4, we determined the kinetics of grafting and the maximum amount of anions that can be attached to the surface. Grafting kinetics were found to be fast: the quantity of benzoxaborolate at the surface of the materials showed little variation between 30 min and 1 week (see supporting information, Figure S4a). Thus, for the rest of the investigations on HA_{ceram}, the grafting time was set to 6 hours. An adsorption isotherm was then plotted by varying the concentration of benzoxaborole in the grafting solution at pH 7.4. As shown in Figure S4b (supporting information), the grafting density progressively increased with the benzoxaborole concentration, but it remained low, below 0.6 molecules/nm^2, despite the excess of benzoxaborole introduced in solution.

Several grafting mechanisms can *a priori* be proposed, based on the following considerations. First, the pH used here for the grafting corresponds the point of zero charge of stoichiometric hydroxyapatite, which is $\sim 7.3 \pm 0.1$ [31], as confirmed for HA_{ceram} by zeta potential measurements ($\zeta < 2$ mV at 25 °C in a 10 mM NaCl aqueous solution). Second, as shown above, only the tetrahedral benzoxaborolate form is present at the surface of HA_{ceram} after the grafting (Figure 2b). Third, given that the pK_a of the benzoxaborole/benzoxaborolate couple is ~ 7.3, both the planar benzoxaborole molecule and the tetrahedral benzoxaborolate anion are present in the initial grafting solution and in the supernatant recovered after the grafting (pH 7.4). This was actually seen by ^{11}B solution NMR spectroscopy, which showed a unique resonance centered at ~ 22 ppm, i.e. at a value intermediate between those of the benzoxaborole and benzoxaborolate counterparts [11,32]. Consequently, the following grafting pathways can be proposed (Figure 3):

- direct coordination of benzoxaborolate anions to surface Ca^{2+} sites, due to a *"local"* attraction of benzoxaborolate anions with the calcium cations at the surface (mechanism A),

- replacement of surface (hydrogen)phosphate anions by benzoxaborolate anions, followed by coordination to nearby Ca^{2+} ions (mechanism B),

- reaction of benzoxaborole molecules with surface hydroxyl groups of HA_{ceram}, leading to the surface-formation of benzoxaborolates, which will then coordinate to Ca^{2+} (mechanism C).

In order to determine the grafting mechanism(s) present, the calcium and phosphorous contents in the grafting supernatants were titrated using ICP-OES. Globally, the phosphorous and calcium concentrations were found to be sensibly the same, whatever the duration of the grafting and whatever the concentration of the grafting solution (see supporting information, Figure S5). These concentrations were in the same order of magnitude as in the control experiment, in which no benzoxaboroles had been introduced in the supernatant. When looking more specifically at the phosphorous, we found that the quantity of phosphates released was well below the amount of benzoxaborolates grafted at the surface of HA_{ceram} (by a factor ~100). Thus an ionic-exchange mechanism like mechanism B (Figure 3) is unlikely. However, both mechanisms A and C can *a priori* explain the grafting of BBzx onto HA_{ceram}. It should be noted that these mechanisms correspond to equilibria occurring during the adsorption, and that the constant remodeling of the hydroxyapatite surface in water (through dissolution/reprecipitation processes for example), as well as the interconversion between benzoxaborole and benzoxaborolate forms in solution, implies that they can take place simultaneously during the grafting at different parts of the surface, depending on the nature of the surface groups exposed locally.

3.1.2 Grafting onto HA_{nano}—Given that stoichiometric hydroxyapatites are not the general form encountered in physiological media, the grafting of benzoxaboroles on a nanocrystalline apatite similar to bone mineral, HA_{nano}, was then studied under more "physiologically relevant" conditions, by working at a pH 7.4 and 37 °C. The experimental conditions used here were similar to those reported for the grafting of bisphosphonate drugs like tiludronate on this type of biomimetic hydroxyapatite [23], and we used the same characterization methodology as for HA_{ceram}. X-ray diffraction characterizations showed the presence of diffraction peaks due to an HA_{nano}-like phase only, and no additional peaks associated with the occurrence of dissolution-reprecipitation processes (Figure 4a). ^{11}B solid state NMR experiments showed that the benzoxaborole is in the form of tetrahedral benzoxaborolate anions (just as for HA_{ceram}), and $^{11}B\{^{31}P\}$ REDOR NMR analyses confirmed the close proximity between these anions and phosphates, and thus the surface grafting onto HA_{nano} (Figures 4b and c).

Titration of the amount of organoboron anions at the surface of the HA_{nano} revealed that irrespective of the duration of the grafting chosen, the amount of benzoxaborolate at the surface of the material was very low, even when using a large excess of molecule in solution (reaching concentrations close to the solubility limit of BBzx). Indeed, based on the experiments performed, a maximum grafting density ~0.08 molecules/nm^2 was found.

The structural features of the HA_{nano} phase differ from those of stoichiometric apatites like HA_{ceram}. HA_{nano} involves a poorly crystalline apatitic core and a "hydrated surface layer" involving loosely bound ions like Ca^{2+} and HPO_4^{2-}, which can be easily exchanged in solution [33]. Grafting onto the HA_{nano} surface may thus imply an ionic exchange with the HPO_4^{2-} anions present within this hydrated layer or coordination to the Ca^{2+} cations

exposed at the surface of the hydrated layer. Titrations of the Ca and P content in solution at the end of the grafting were carried out, showing that they were in the same order of magnitude as in the control experiment (~10 mg/L for Ca and ~100 mg/L for P). Hence, in contrast with what has been observed for the grafting of bisphosphonates on biomimetic or calcium deficient apatites [23,34], no ionic exchange occurred here. It can therefore be suggested that the grafting occurs by coordination of benzoxaborolates to calcium sites exposed at the periphery of the hydrated layer, through a mechanism that is similar to mechanism A.

3.2 Comparison of the grafting of benzoxaborolates on HA$_{ceram}$ with that of other oxoanions

Given that in physiological media, benzoxaborol(at)es will be in competition with other anions, including oxoanionic groups belonging biomolecules, the affinity of benzoxaborolates with hydroxyapatite surfaces was compared to that of carboxylate and organophosphate groups. In order for the binding to occur through the oxoanionic functions in carboxylates and organo-phosphates, molecules bearing a simple phenyl group were studied here, i.e. phenylphosphate (C_6H_5-OPO_3^{2-}, noted PhP) and benzoate (C_6H_5-COO^-, noted PhC). Moreover, the HA$_{ceram}$ phase was used as a model material for these tests, due to the higher grafting density at its surface.

The grafting kinetics onto HA$_{ceram}$ were similar for PhP and PhC compared to BBzx (Figure S6, supporting information) [35]. Grafting experiments were thus performed under similar conditions for the 3 molecules (BBzx, PhC and PhP), i.e. using the same pH (pH 7.4), the same reaction time (6 h), and the same initial concentration of molecule in solution (~17 mmol.L^{-1}). As shown in Figure 5, the amount of PhC grafted is only slightly higher than for BBzx, while in the case of PhP the grafting density is more than twice as high. Such differences can be explained by looking at the Ca and P content in the supernatant at the end of the grafting. Indeed, while the Ca and P concentrations determined for PhC are in the same range as those measured for BBzx (~9 mg/L for Ca, and ~ 0.7 mg/L for P), the P concentration in the medium after reaction with PhP was found to exceed 400 mg/L. This suggests that while PhC attaches by simple coordination to the surface Ca^{2+} sites (in a way similar as in mechanism A, Figure 3), PhP attaches by displacement of surface phosphates (like in mechanism B). The fact that phenylphosphate has two negative charges at pH 7.4 plays in favor of this ionic-displacement mechanism, leading to a stronger anchoring and higher affinity of the anion in the apatite surface, in comparison to the mono-charged benzoxaborolate and benzoate anions. It is worth noting that the grafting mechanism proposed here for PhP recalls previously published observations on the attachment of (bis)-phosphonates onto hydroxyapatite surfaces, in which the ionic displacement of surface phosphates was observed [34,36]. Concerning PhC, our results are also in line with previous studies on grafting of carboxylate groups on hydroxyapatite, which had suggested that coordination to surface Ca^{2+} sites occurs (without any ionic displacement) [18,37].

3.3 Grafting of the 3-piperazine-bis(benzoxaborole) molecule onto hydroxyapatite

Studies similar to those reported above on the simplest benzoxaborole motif BBzx were then performed with a more complex benzoxaborole, bis-pipe-BBzx, that was recently described

for its antimicrobial and antifungal properties (Figure 1d). In this case as well, it was found that the more complex benzoxaborole molecule is present at the surface of HA_{ceram} under the tetrahedral benzoxaborolate form (Figure 6a), that no secondary crystalline phases precipitate during the reaction (Figure S7a, supporting information), and that the amount of molecule grafted was low (less than $1\%_{wt}$ of bis-pipe-BBzx present in the grafted material).

Given the low grafting densities observed, we then attempted to increase the amount of molecules attached to the surface by synthesizing the hydroxyapatite phase by precipitation in the presence of bis-pipe-BBzx molecules in solution. Although higher boron concentrations could indeed be achieved through this new synthetic procedure (referred to as "*in situ*" grafting), additional peaks were observed on the ^{11}B NMR spectra that are likely associated with the degradation of bis-pipe-BBzx (Figure 6b). Indeed, our previous work on the intercalation of BBzx into LDH had shown that the confinement of benzoxaborolate anions inside the alkaline sheets of the LDH could lead to their degradation and to the formation of by-products like borate. Here, the position of these new ^{11}B resonances is the same as observed when precipitating hydroxyapatite in presence of borates instead of bis-pipe-BBzx (Figure 6b). The same observations were made when working with the simplest BBzx molecule (Figure S8). Hence, this second synthetic strategy appears as unsuitable to the preparation of apatite formulations for benzoxaboroles.

4. Discussion

Benzoxaboroles are a family of molecules that are finding increased applications in the biomedical field, particularly as a "privileged scaffold" for the design of new drugs. Here we show that benzoxaboroles can be grafted onto hydroxyapatite phases like HA_{ceram} and HA_{nano} in water at physiological pH, and that these molecules are present at the surface as benzoxaborolate anions only. The grafting is fast (completed in a few hours), but the amount of benzoxaborolate adsorbed remains low, with less than 0.6 molecules per nm^2 for BBzx on HA_{ceram}, and less than 0.1 molecules per nm^2 for BBzx on HA_{nano}. This grafting density is well beneath the ~5 molecules/nm^2 observed upon the formation of a dense monolayer of phenylphosphonic acid (C_6H_5-PO(OH)$_2$) at the surface of inorganic materials [29], and also much lower than what had been reported for the grafting of bisphosphonates like tiludronate onto biomimetic apatites [23].

Several explanations can be proposed to explain such low grafting densities:

i. The similarity between the point of zero charge of hydroxyapatite (~7.3 ± 0.1)[31] and the pK_a of the benzoxaborole/benzoxaborolate couple (~7.3) [1] implies that no significant long-distance attractive ionic interactions will occur between the benzoxaborolate anions in solution and the HA surface during the grafting. Moreover, the neutral benzoxaborole form is expected to only weakly interact with the apatite surface through H-bonding or van der Waals interactions.

ii. Benzoxaborolates only have one negative charge, and hence they will have a lower affinity for the HA surface than any HPO_4^{2-} anions leached upon immersion of the HA phases in water at pH 7.4. This is especially true in

the case of HA_{nano}, because the phosphate concentration in solution after simple immersion of this nanocrystalline apatite is higher, and that monovalent anions (like BBzx) have a lower affinity for the "hydrated amorphous layer" present at the surface of such apatites than divalent ones (like HPO_4^{2-}).

iii. All benzoxaborolates may not be attached perpendicularly to the apatite surface (with the aromatic ring pointing outwards) and some may also be lying flat and/or in a more tilted configuration with respect to the surface. In the case of HA_{ceram}, the absence of plateau on the adsorption isotherm (Figure S4b, supporting information) actually implies that the mode of binding of the benzoxaborolates at the HA_{ceram} surface may vary with the benzoxaborole concentration in solution.

We also report on the attempt to increase the amount of organoboron molecule in the material using an "*in situ*" grafting protocol, in which the surface-attachment of benzoxaborolates occurs during the precipitation of the hydroxyapatite. However, as illustrated for BBzx and bis-pipe-BBzx, this may be causing the degradation of the molecule (Figure 6b and S8), and should therefore be avoided for formulation purposes. In contrast, the post-grafting onto phases like HA_{ceram} or HA_{nano} is suitable to keep the benzoxaborolate species intact. However, it should be noted that the amount of molecules present in the material will be low (less than ~1 wt %), and that similar to our previous work on LDH-BBzx compounds [11], the best strategy to preserve the BBzx-grafted HA materials for long periods of time is to store them in a freezer, as these phases tend to slowly evolve upon storage at room temperature over several months (Figure S9, Supporting Information). An alternative approach to increasing the amount of benzoxaborole grafted at the surface of apatites may consist in prefunctionalizing the apatite phase with a small molecule which will then interact with the benzoxaboroles, following the same general idea as recently proposed for adsorbing proteins on apatites [38]. For example, the pre-grafting of a small molecule exposing an amine function (which would be under the positive ammonium form at pH 7.4), would allow creating an overall positive charge at the HA surface, and thereby favor the grafting of the benzoxaborolate anions in solution, thanks to attractive electrostatic forces. However, this strategy has not been investigated yet and would deserve to be explored on its own.

Under physiological conditions, it can be expected that benzoxaborole molecules, directly grafted onto the surface of biological hydroxyapatites, will very quickly get desorbed, due to competition with various anionic species. First, as mentioned above, the phosphate anions present in body fluids will have a higher affinity with the apatite surfaces, and since their concentration in body fluids is high, they will easily replace any grafted BBzx species. In this context, it is worth mentioning that we found that a single washing of the BBzx-grafted HA_{ceram} material for 10 minutes with PBS is sufficient to remove all organoboron species from the surface (see experimental section). Second, benzoxaborol(at)es will also be in competition with other organic anions commonly found in biomolecules, like carboxylates and organophosphates, which have similar to higher affinities for apatite surfaces (Figure 5),

and which will also be present in the medium at much higher concentrations than the organoboron molecules.

Overall, these results imply that benzoxaborole drugs administered using oral or parenteral routes are unlikely to remain adsorbed onto mineral surfaces like hydroxyapatite for long periods of time, meaning that their biodistribution will not be affected by such phenomena. This is an important conclusion not only for the benzoxaborole drugs currently being developed, but also for other biomedical applications of these molecules, in which they being used as sugar-targeting agents for the internalization of proteins in cells [7,39]. Moreover, it means that the formulation of benzoxaborole-based molecules by grafting onto calcium phosphates like hydroxyapatite will most probably lead to a fast (burst) release, which can nevertheless be subsequently tuned through the preparation of apatite/biopolymer composites using a strategy that is described in our recent work on PLLA-LDH formulations of benzoxaboroles [12].

5. Conclusion

In this work, we have shown the importance of carrying out prospective studies on the privileged benzoxaborole scaffold (BBzx) in order to determine how the presence of this organoboron motif in therapeutic molecules may affect their reactivity in physiological conditions (in particular in contact with biominerals like calcium phosphates), and what are the best synthetic approaches for developing new formulations for these species, whose reactivity at the interface of common biomaterials like hydroxyapatites remains unexplored. Through several different investigations on the grafting of benzoxaboroles onto hydroxyapatite phases, we demonstrate here that the BBzx scaffold has a poor affinity for apatite surfaces. However, despite the poor affinity, benzoxaboroles can be weakly attached onto apatite surfaces under the tetrahedral benzoxaborolate form, and this may be useful for developing specific apatite-containing formulations or biomaterials involving these molecules. Furthermore, the synthesis and characterization methodologies, described herein, will be applicable to the grafting of benzoxaborole drugs onto other calcium phosphate phases (crystalline or amorphous) that currently being developed/used for biomedical applications [13,40,41]. On a more general perspective, it can be expected that the conclusions obtained in this work on benzoxaboroles will also remain valid for the parent organoboron molecules, boronic acids, which are increasingly being used in the biomedical context, and notably in biomolecules, nanoparticles and biopolymers [42,43].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of significance

Benzoxaboroles are an emerging family of molecules which have attracted much attention in the biomedical field, notably for the design of new drugs. However, the way in which these molecules, once introduced in the body, may interact with bone mineral is still unknown, and the possibility of associating benzoxaboroles to calcium phosphates for drug-formulation purposes has not been looked into. Here, we describe the first study of the adsorption of benzoxaboroles on hydroxyapatite, which is the main mineral phase present in bone. We describe the mode of grafting of benzoxaboroles on this material, and show that they only weakly bind to its surface, especially in comparison to other ionic species commonly found in physiological media, such as phosphates and carboxylates. This demonstrates that administered benzoxaborole drugs are unlikely to remain adsorbed on hydroxyapatite surfaces for long periods of time, which means that their biodistribution will not be affected by such phenomena. Moreover, this work shows that the formulation of benzoxaborole drugs by association to calcium phosphates like hydroxyapatite will lead to a rapid release of the molecules.
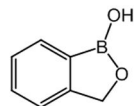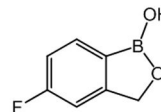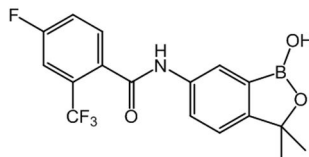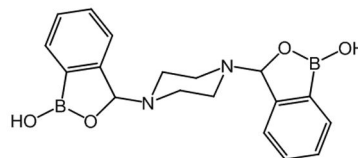
Fig. 1.

Representation of the benzoxaborole motif BBzx (a), and of benzoxaborole molecules with therapeutic properties against fungi (b and d) or trypanosomes (c) [6,10].
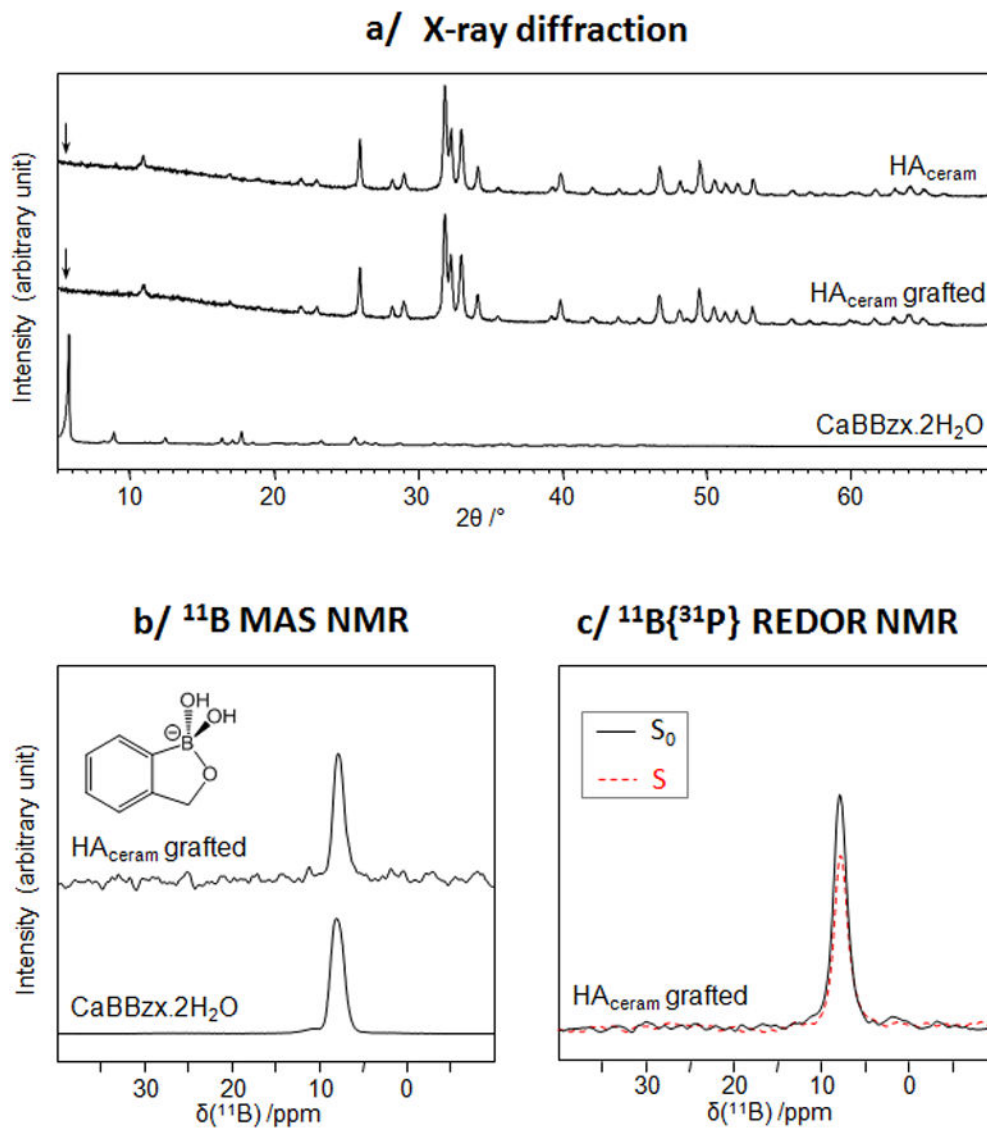
a/ X-ray diffraction

b/ ^{11}B MAS NMR

c/ ^{11}B{^{31}P} REDOR NMR

Fig. 2.
Characterization of the BBzx-grafted HA$_{ceram}$ phase and of related materials using XRD (a), ^{11}B MAS NMR (b) and ^{11}B{^{31}P} REDOR NMR (c).
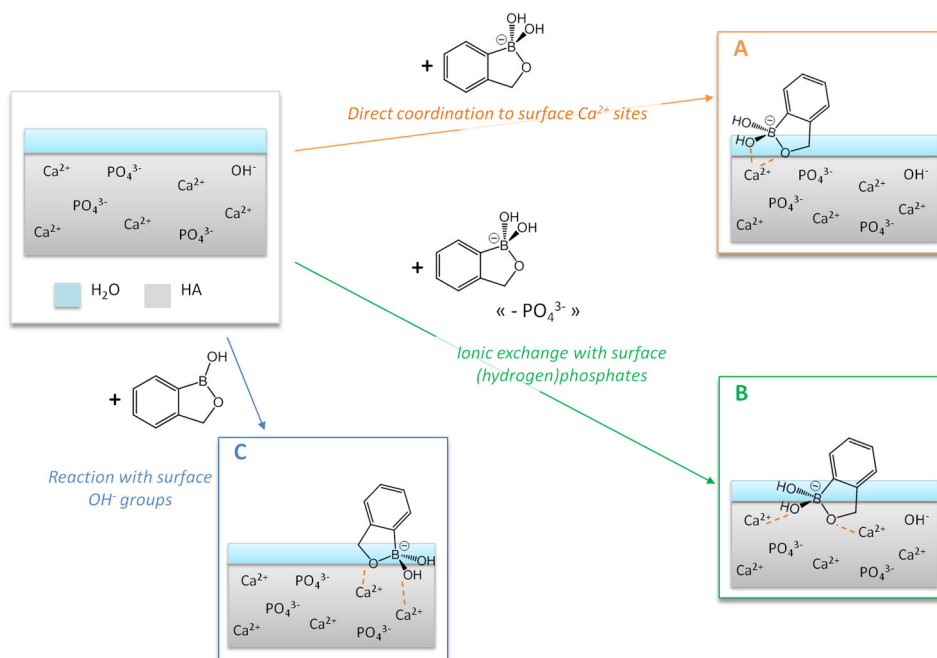
Fig. 3.

Schematic representation of possible grafting mechanisms of benzoxaboroles at the surface of HA$_{ceram}$. For simplicity, the HA$_{ceram}$ phase is depicted in grey, with some of the ions involved in the grafting process, while the layer of water molecules more closely bound to the surface is shown in blue.
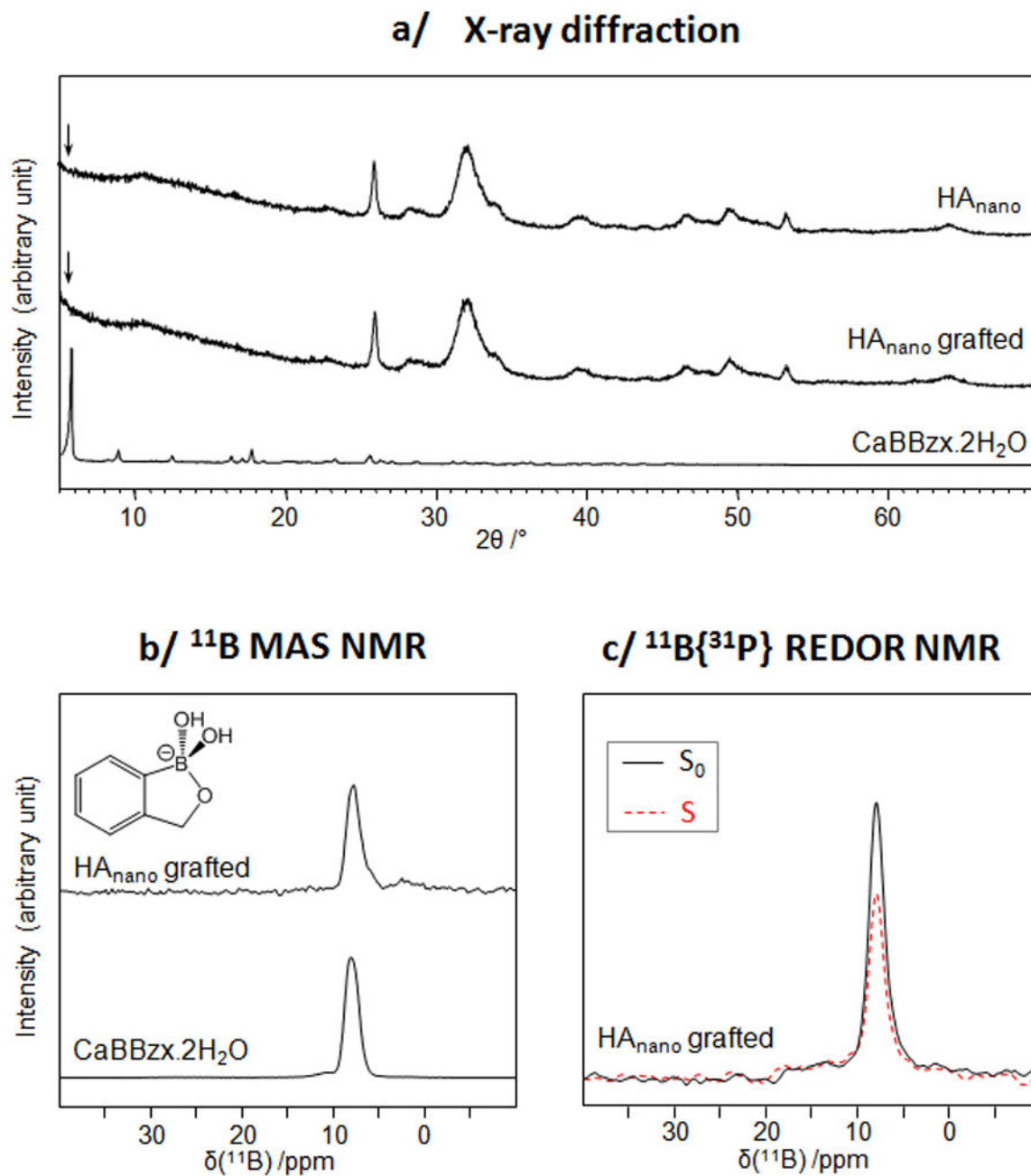
Fig. 4.
Characterization of the BBzx-grafted HA_{nano} phase and of related materials using XRD (a), ^{11}B MAS NMR (b) and $^{11}B\{^{31}P\}$ REDOR NMR (c).
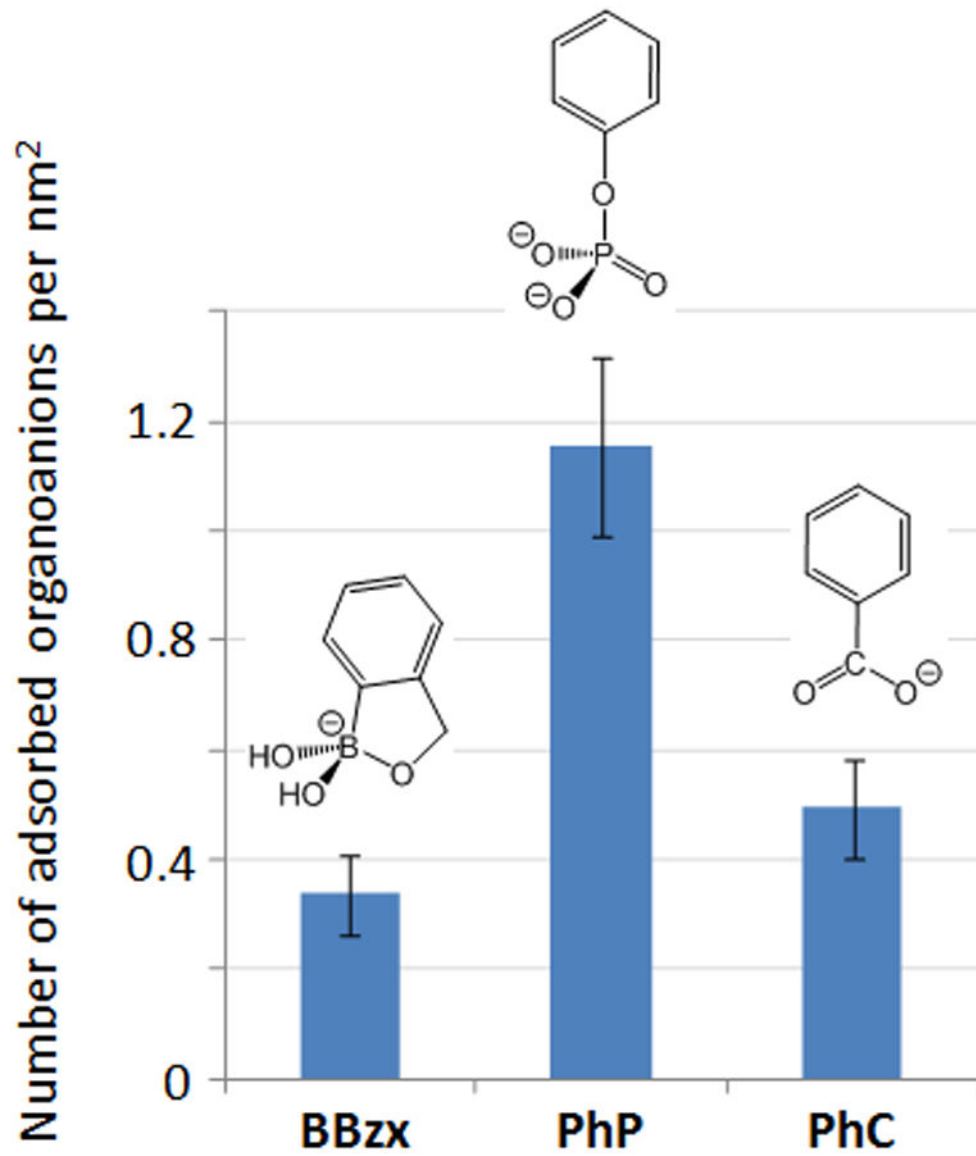
Fig. 5.
Comparison of the grafting density of benzoxaborolate, phenylphosphate and benzoate anions at the surface of HA_{ceram}.
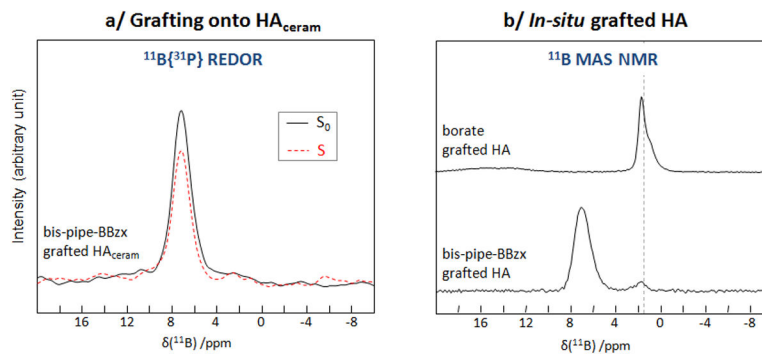
Fig. 6.
Characterizations of bis-pipe-BBzx-grafted hydroxyapatite phases: ^{11}B$\{^{31}$P$\}$ REDOR NMR of the grafted HA$_{ceram}$ material (a); and ^{11}B MAS NMR of an "*in-situ*" grafted HA material in comparison to a borate-grafted HA phase obtained under similar conditions (b). In the spectrum of the borate-grafted HA, the broad weak resonance between ~20 and ~12 ppm is due to traces of boric acid.