Synthesis of hybrid polyphenol/hydroxyapatite nanomaterials: adsorption *vs.* *in situ* incorporation

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Abstract

Plant-derived natural bioactive molecules are of great therapeutic potential but their application in nanomedicine has been so far scarcely studied. This work aimed at comparing two methodologies, *i.e.* adsorption and *in situ* incorporation, to prepare hybrid polyphenol/hydroxyapatite nanoparticles. Two flavonoids, baicalin and its aglycone derivative baicalein, and two phenolic acids derived from caffeic acid, rosmarinic and chlorogenic acids, were studied. Adsorption of these polyphenols on pre-formed hydroxyapatite nanoparticles did not modify particles size or shape and loading was less than 10 % (w/w). In contrast, presence of polyphenols during the synthesis of nanoparticles significantly impacted, and sometimes fully inhibited, hydroxyapatite formation, but recovered particles could exhibit higher loadings. Antioxidant properties of the polyphenols were preserved after adsorption but not when incorporated *in situ*. These results provide fruitful clues for the valorization of natural bioactive molecules in nanomedicine.

1. Introduction

After many years of use of synthetic or hemi-synthetic molecules in the pharmaceutical area, attention has recently turned towards natural molecules, mainly extracted from plants, that exhibit many advantages. For example, polyphenols are secondary metabolites widespread in plants where they play a role of defence against pathogens.^{1,2} They can be separated in two groups, flavonoids and non-flavonoids. Flavonoids are composed of a carbon skeleton of two benzene rings A and B linked by a linear three-carbon chain that forms a closed pyran ring (ring C) with A benzene ring. Among them, baicalin (BA) and its aglycon derivative baicalein (BE) (**Fig. 1a,b**) are extracted from the root of the plant *Scutellaria baicalensis* and used in the Chinese traditional medicine, mainly as herbal tea. BA and BE have been shown to exhibit anti-inflammatory, antioxidant, antimicrobial and anticancer properties.³⁻⁸ Non-flavonoids polyphenols are phenolic acids such as derivatives of caffeic acid like rosmarinic acid (RA) and chlorogenic acid (CA) (**Fig. 1c,d**). CA is an ester of caffeic acid with (L)-quinic acid, and is found in many foods and beverages, mainly coffee, fruits, vegetables and lamiaceae (such as oregano, peppermint and thyme).^{9,10} It is known for its antioxidant and antibacterial properties.¹¹⁻¹³ Rosmarinic acid is an ester of caffeic acid with 3-(3,4-dihydroxyphenyl) lactic acid found in many culinary herbs like rosemary, with anticancer, antimicrobial and antioxidant properties.¹⁴⁻¹⁶

Hydroxyapatite nanoparticles (HAp NPs) are being developed for a wide range of medical applications,^{17,18} and especially for bone regeneration.^{19,20} They are often associated with bioactive compounds, such as antioxidants,^{21,22} osteogenic factors,^{23,24} anti-inflammatory drugs,^{25,26} antimicrobial agents^{27,28} or antifibronolytics.²⁹ The two main methods to combine bioactive molecules and HAp NPs are adsorption on pre-formed particles or incorporation during their synthesis. In the case of adsorption, the loading rate is controlled by the interactions between the active component and the hydroxyapatite surface.³⁰⁻³³ For example, it is well-

known that molecules such as bisphosphonates have a strong affinity for hydroxyapatite surface.^{23,24} Other functional groups like carboxylic acids or phenols have also been shown to bind hydroxyapatite surfaces,^{34,35} allowing for the efficient adsorption of natural and synthetic antibiotics or anti-inflammatory molecules.^{25,36} Synthesis of HAp in the presence of polyphenols, such as gallic acid or tannic acid,^{37,38} has also been reported but led to a partial or complete inhibition of hydroxyapatite crystallization, which was attributed either to complexation of Ca^{2+} or adsorption on the surface of hydroxyapatite seeds. However, to our knowledge, these two approaches were never compared using same polyphenols at comparable concentrations and, in particular, their relative benefits in terms of hydroxyapatite formation and molecule loading were not studied so far.

Fig.1. Molecular structures of (a) baicalin BA, (b) baicalein BE, (c) chlorogenic acid CA and (d) rosmarinic acid RA.

For this purpose, we selected four polyphenols of interest, BA, BE, CA and CE, belonging either to the flavonoid or non-flavonoid family, in an aglycon or glycosylated form. HAp NPs were synthesized through a wet method in aqueous medium and polyphenols were

incorporated either after synthesis or by addition to the HAp precursor calcium solution. Incorporation during synthesis achieved a higher quantity of loaded molecules in the recovered NPs but at the expense of hydroxyapatite formation, that was totally inhibited when RA was initially present in large quantity. Lower loadings could be reached through adsorption without any significant modification of the HAp NPs structure nor morphology. For both methods, BE was found to be the molecule with the highest incorporation rates. Evaluation of the antioxidant properties of the NPs evidenced that the adsorption process preserves the initial activity of the polyphenols whereas the *in situ* approach fully inhibited it.

2. Experimental Section

2.1. Chemicals

Chlorogenic acid (CA) and rosmarinic acid (RA) was purchased from Carbosynth Limited (Compton, Berkshire, UK). Baicalein (BE) and baicalin (BA) were purchased from TCI Europe (Zwijndrecht, Belgium). Stock solutions of the four molecules (0.1 M) were prepared in dimethylsulfoxide (DMSO, Sigma Aldrich)

2.2. Hydroxyapatite synthesis

Pure hydroxyapatite was synthesized by co-precipitation from calcium hydroxide and ammonium dihydrogenophosphate solutions.³⁴ Briefly, $\text{Ca}(\text{OH})_2$ (7.4 g) was suspended in 100 mL of distilled water during 1.5 h. Then 50 mL of an aqueous solution of $\text{NH}_4\text{H}_2\text{PO}_4$ (1.2 M) was slowly added to the calcium hydroxide and the mixture was left to react for 48 h at 25 °C under stirring. The resulting particles were recovered by centrifugation at 5000 rpm for 5 minutes, washed three times with distilled water and dried at 100 °C for 24 h.

2.3. Hybrid particle synthesis

2.3.1. Adsorption method

For adsorption experiments, the polyphenol concentration was set to 0.05 mM, a value close to the apparent solubility of BA and BE (*ca.* 0.1 mM) in distilled water. The hydroxyapatite particle weight was then calculated to obtain a polyphenol:Ca molar ratio of 1:1 in the reaction mixture. On this basis, 10 mg of HAp NPs were suspended in 200 mL of the 0.5 mM polyphenol solution and stirred at room temperature for 24 h. Particles were then recovered by centrifugation and dried at 100 °C for 24 h.

2.3.2. In situ incorporation

In these experiments, two polyphenol concentrations, 0.05 mM and 0.5 mM, were tested. To avoid too small polyphenol:Ca ratios, initial concentrations of $\text{Ca}(\text{OH})_2$ and $\text{NH}_4\text{H}_2\text{PO}_4$ were decreased by a factor of 100 compared to the above-described protocol. On this basis, polyphenols were dissolved in 100 mL of a 10 mM calcium hydroxide solution for 1.5 h before addition of 50 mL of a 12 mM ammonium dihydrogenophosphate solution. After stirring at room temperature for 24 h, particles were recovered by centrifugation and dried at 100 °C for 24 h.

2.4. Characterization methods

2.4.1. Thermogravimetry analysis (TGA)

Thermogravimetric analysis (TGA) was performed with an SDT Q600 apparatus from TA Instruments, between 25 °C and 700 °C, with a ramp of 10 °C.min⁻¹, under air.

2.4.2. Power X-Ray Diffraction (XRD)

X-Ray diffractograms were recorded on a D8 diffractometer (Bruker), equipped with a $\text{CuK}\alpha$ source (1.54 Å) and a Bragg-Brentano $\theta - 2\theta$ geometry, between 7 ° and 80 ° with a variable slit V12, a step of 0.03 ° and a scan speed of 1 s/point, except for the diffractogram of HAp obtained in diluted conditions that was recorded with a fast detector LynxEye (Bruker) with a variable slit V12 and an exposition time of 1.67/step/canal. Crystallite sizes were calculated

using the Scherrer equation. The diffraction peak at $2\theta = 26^\circ$, corresponding to the plan (002), was used for the calculation due to its adequate resolution and absence of overlap with other peaks.

2.4.3. Transmission Electron Microscopy (TEM)

Nanoparticles were deposited on 300 square mesh carbon-coated copper grids (Ted Pella, Redding, CA) and observed with a Tecnai spirit G2 microscope (Thermofischer Company, FEI Company, Philips, Netherlands) operating at 120 kV equipped with a Gatan Orius 1000 camera.

2.4.4. Nuclear Magnetic Resonance (NMR) spectroscopy

Solid-state Nuclear Magnetic Resonance (NMR) experiments were performed on a Bruker Avance III spectrometer operating at 300.3, 121.6 and 75.5 MHz for ^1H , ^{31}P and ^{13}C , respectively. A broadband dual resonance 4 mm probe was used with Magic Angle Spinning (MAS) at $\nu_r = 11.962$ kHz. ^1H experiments were acquired with a 90° pulse, a Relaxation Delay $\text{RD} = 2$ s, and a Number of Scans $\text{NS} = 4$. $\{^1\text{H}\}\text{-}^{31}\text{P}$ spectra were obtained using Cross-Polarization (CP) with a contact time $t_{cp} = 1$ or 10 ms, $\text{RD} = 1$ s and $\text{NS} = 128$. For $\{^1\text{H}\}\text{-}^{13}\text{C}$ experiments, CP was used with $t_{cp} = 2$ ms, $\text{RD} = 1$ s and $\text{NS} = 47752$. SPINAL-64 decoupling¹ was applied at $\nu_{IH} = 70$ kHz. Spectra were referenced to TMS for ^1H and ^{13}C , and to H_3PO_4 at 85% for ^{31}P .

2.5 Antioxidant activity assays

The antioxidant property of the polyphenols, in solution or within the hybrid nanoparticles, was evaluated by their radical scavenging activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) following a modified Blois method.^{39,40} Solutions of polyphenols (final concentration 5 μM) or suspensions of 5.0 ± 0.5 mg of the nanoparticles in a TRIS buffer (pH 7.4) were mixed with an equal volume of DPPH solution in ethanol (100 μM). Samples were left to react 20 min in the dark, centrifuged when required, and absorbance (A) was measured at 517 nm using a Uvikon

XS spectrophotometer. Absorbance (A_{ref}) of a 1:1 mixture of TRIS and DPPH at the same wavelength was used as the reference. Bleaching rate was calculated as:

$$\text{Bleaching rate (\%)} = 100 \times [1 - (A/A_{ref})] \quad (1)$$

All experiments were performed as duplicates.

3. Results and discussion

3.1 Hydroxyapatite nanoparticles

Hydroxyapatite nanoparticles (HAp NPs) were synthesized following a wet method by co-precipitation of calcium hydroxide and ammonium dihydrogenophosphate.³⁴ The yield of synthesis was 90 % based on weight measurements. TEM observation indicated that particles were plate-like shaped, 31 ± 6 nm in length and 9 ± 3 nm in width (**Fig. 2a**). Specific surface area calculated from N₂ sorption data using the BET method was 160 m².g⁻¹ (**Fig. S1** in ESI). The X-ray diffractogram confirmed the formation of hydroxyapatite (**Fig. 2b**).

Fig. 2. (a) TEM image and (b) X-ray diffractogram of synthesized HAp NPs.

3.2 Hybrid nanoparticles

3.2.1 Adsorption on HAp NPs

The quantity of adsorbed polyphenols was measured by thermogravimetric analysis (**Table 1** and **Fig. S2** in ESI). BE was the most efficiently adsorbed polyphenol, with 63 mg per g of hybrid particles, loadings in RA and CA were about two times smaller and BA exhibited an intermediate adsorption value (37 mg.g⁻¹). When calculated as the molecular surface density, polyphenol loading is again the highest for BE while similar results are obtained for the three other molecules.

Table 1 Quantity of adsorbed polyphenol on HAp measured by TGA, particle dimensions as obtained from TEM and crystallite size from XRD.

Sample	Incorporated quantity (mg.g ⁻¹)	Incorporated quantity (molecules.nm ⁻²)	Particle length/width (nm)	Crystallite size (nm)
HAp-RA	31	0.3	31 ± 10/8 ± 2	21 ± 5
HAp-CA	27	0.3	43 ± 11/12 ± 1	23 ± 5
HAp-BA	37	0.3	27 ± 4/8 ± 3	24 ± 5
HAp-BE	63	0.9	33 ± 10/15 ± 3	24 ± 5
HAp	-	-	31 ± 6/9 ± 3	21 ± 2

TEM image analyses did not evidence modification of particles dimension after adsorption (**Fig. 3a-d** and **Table 1**). X-Ray diffractograms did not indicate the formation of additional mineral phases (**Fig. 3e**) and crystallite sizes, as estimated by the Scherrer formula, were similar to the one before adsorption (**Table 1**).

Fig. 3. TEM image of hydroxyapatite after adsorption of (a) rosmarinic acid, (b) chlorogenic acid, (c) baicalin and (d) baicalein; and (e) superimposition of X-ray diffractograms of HAp NPs before and after adsorption of polyphenols. The observed decrease in signal-to-noise ratio in presence of BA, BE and CA can be attributed to the low quantity of powder used for the XRD measurement.

For further characterization, ^1H , $\{^1\text{H}\}\text{-}^{13}\text{C}$ and $\{^1\text{H}\}\text{-}^{31}\text{P}$ solid-state MAS NMR experiments were performed before and after adsorption of polyphenols. Superimposition of

^1H spectra is presented in **Fig. 4a**. On the ^1H spectrum of pure HAp, the signal at 0 ppm, characteristic of protons in the hydroxyapatite crystal lattice, could be noticed, together with a broader peak at 5.5 ppm which can be ascribed to physisorbed water.⁴¹ Similar spectra were obtained for HAp-RA, HAp-CA and HAp-BA, except that the peak at 5.5 ppm was broader. For HAp-BE however, this peak was not clearly identified. Instead, a broad band was observed at 7.2 ppm, that can be attributed to the aromatic ring of this molecule.

Spectra obtained by $\{^1\text{H}\}$ - ^{13}C CP experiments are presented on **Fig. 4b**. A residual rotor background signal was present as shown in gray on bottom spectrum. All spectra were characterized by a broad peak of weak intensity at 170 ppm ascribed to carbonates species (**Fig. 4b**). This band is dissymmetrical, which suggests a type B substitution, *i.e.* a substitution of phosphates groups by carbonates groups in the crystal lattice of hydroxyapatite.⁴² In addition, an intense peak was visible at approximately 128 ppm on the HAp-BE spectrum, and, to lesser extent, on the one of HAp-BA. This band corresponds to aromatic carbons of baicalein and baicalin.

$\{^1\text{H}\}$ - ^{31}P CP spectra at $t_{cp} = 1$ and 10 ms are presented on **Fig. 4c i)** and **ii)**, respectively. At $t_{cp} = 1$ ms, both crystalline (narrow) and amorphous (broad) components were visible at the same chemical shift of 2.8 ppm. For all samples, adsorption leads to a broadening of this peak, highlighting an increase in the relative amount of the amorphous component. At longer contact time ($t_{cp} = 10$ ms), all spectra were identical, showing no significant modification of the structure of the core of HAp particles.

Fig. 4 Solid-state NMR experiments performed on pure HAp, HAp-CA, HAp-RA, HAp-BA and HAp-BE (initial concentration for adsorption = 0.05 mM) (* indicates residual peaks of acetone and ethanol): (a) ^1H , (b) $\{^1\text{H}\}$ - ^{13}C CP and (c) $\{^1\text{H}\}$ - ^{31}P CP spectra at t_{cp} = i) 1 and ii) 10 ms.

3.2.2 *In situ* incorporation

In a first step, it was first necessary to check whether the use of lower calcium hydroxide and ammonium dihydrogenophosphate concentrations impacted HAp particles structure. XRD evidenced that although hydroxyapatite was the major recovered phase, an additional peak at *ca.* $2\theta = 18^\circ$ was present. (**Fig. 5a**). This diffraction peak could be assigned to the (001) diffraction peak of $\text{Ca}(\text{OH})_2$, although this assumption could not be fully confirmed since no other peak of $\text{Ca}(\text{OH})_2$ was observed, probably due to the overlap between $\text{Ca}(\text{OH})_2$ and HAp patterns for all other calcium hydroxide peaks.

In the presence of polyphenols at 0.05 mM, formation of hydroxyapatite was assessed by XRD (**Fig. 5a**). Only peaks assigned to HAp were observed in the presence of BA and CA while the additional peak at *ca.* $2\theta = 18^\circ$ was also visible when RA or BE were used as additives. The calculated crystallite size was similar for all samples. **Fig. 5b-e** display TEM images of obtained hybrid particles. HAp-CA and HAp-BA consisted of platelet-shaped particles, similar to pure HAp, but apparently more aggregated. HAp-RA and HAp-BE particles had a less defined shape (**Fig. 5b** and **e** respectively). The length of the particles, as measured from TEM images, was reduced from 30 ± 7 nm for pure HAp to 14 ± 2 nm for HAp-RA, 19 ± 2 nm for HAp-CA, 13 ± 2 nm for HAp-BA and 17 ± 3 nm for HAp-BE (**Table 2**). In parallel, particle width remained unchanged compared to pure HAp NPs (8 ± 4 nm) in the presence of CA and BE but significantly decreased with RA and BA (4.2 ± 0.5 nm) (**Table 2**).

Fig. 5. (a) XRD patterns of hydroxyapatite particles synthesized in the presence of 0.05 mM of rosmarinic acid (HAp-RA), chlorogenic acid (HAp-CA), baicalin (HAp-BA) and baicalein (HAp-BE); and their TEM images: (a) HAp-RA, (b) HAp-CA, (c) HAp-BA and (d) HAp-BE

The presence of polyphenols also influenced the yield of synthesis, as calculated from the mass of dry solid, from *ca.* 65 % for HAp NPs alone, to 45-55 % in the presence of the four molecules (**Table 2**). TGA measurements indicated that polyphenol loading was 52 mg.g⁻¹ for RA and BE, 42 mg.g⁻¹ for CA, and 54 mg.g⁻¹ for BA (**Table 2** and **Fig. S3** in ESI). The final

polyphenol:calcium molar ratio in the hybrid particles ranges between 0.01 for HAp-BA and 0.02 for HAp-BE, thus higher than in the initial solution (0.0075) (**Table 2**).

Table 2. Synthesis of HAp NPs in presence of polyphenols at 0.05 mM and 0.5 mM: yield, particles dimensions as measured from TEM, crystallite size calculated from XRD data, incorporated quantity measured from TGA and estimated final polyphenol: Ca molar ratio.

Sample	Initial concentration of polyphenol (mM)	Yield (%)	Particle length/width (nm)	Crystallite size (nm)	Incorporated quantity (mg.g⁻¹)	Final molar ratio polyphenol : Ca
HAp	-	66	30±7/8±4	15±3	-	-
HAp-RA	0.05	46	14±2/4.2±0.5	16±1	52	0.015
	0.5	-	-	-	-	-
HAp-CA	0.05	56	19±2/6±2	15±1	42	0.011
	0.5	43	-	14±2	73	0.02
HAp-BA	0.05	46	13±2/4.2±0.5	13±2	54	0.012
	0.5	50	-	18±2	111	0.025
HAp-BE	0.05	53	17±3/7±1	17±1	52	0.02
	0.5	42	-	14±3	135	0.05

A second set of experiments were performed at a 0.5 mM polyphenol concentration in an attempt to further increase the polyphenol-to-calcium ratio in the particles. Such an increase in the initial concentration of BA and BE was possible because of their enhanced solubility at pH 12 (compared to the adsorption conditions performed in deionized water). XRD patterns presented in **Fig. 6a** show that hydroxyapatite was the main precipitated mineral phase in the

presence of CA, BE and BA. Calculated crystallite sizes were similar to the ones calculated for NPs synthesized in the presence of 0.05 mM of polyphenols, *ca.* 15 nm (**Table 2**).

In contrast, synthesis of NPs in the presence of 0.5 mM RA was unsuccessful. The particle washing step in distilled water led to the dissolution of the solid phase obtained by co-precipitation so that almost no sample could be harvested. In order to recover few mg of material to perform XRD analysis, the washing step was skipped for this sample. In this case, the X-Ray diffractogram did not show peaks corresponding to the hydroxyapatite structure but comparison of experimental data with database showed that the pattern could be assigned to the calcium carbonate (CaCO_3) calcite phase. HAp formation was thus totally inhibited by the presence of rosmarinic acid. Therefore, this compound was referred as CaP (for calcium phase)-RA. The identification of CaP-RA as calcite is consistent with its solubilization during washing, as calcite is known to be quite soluble in water near neutral pH.⁴³ The morphology of CaP-RA was found to be very different from other HAp NPs since particles were larger and rectangular-shaped whereas HAp-BE particles were platelet-like shaped (**Fig. 6b** and **6c**, respectively). It is worth noticing that the main diffraction peak of calcite ($2\theta = 29^\circ$) is also visible on the diffractogram of HAp-BE, and to a lesser extent for HAp-CA but not for HAp-BA.

Fig. 6. (a) XRD pattern of hydroxyapatite particles synthesized in the presence of 0.5 mM of rosmarinic acid (CaP-RA), chlorogenic acid (HAp-CA), baicalin (HAp-BA) and baicalein (HAp-BE), compared with patterns of hydroxyapatite (JCPDS 09-0432) and calcite (JCPDS 05-0586); TEM images of (b) HAp-BE and (c) CaP-RA.

For HAp-CA and HAp-BE NPs, the apparent yield of HAp synthesis in the presence of a 0.5 mM polyphenol initial concentration was lower than with 0.05 mM, while it was quite similar for HAp-BA whatever the concentration (**Table 2**). The amount of incorporated polyphenols in the hybrid particles was 73 mg.g⁻¹ for CA, 135 mg.g⁻¹ for BE and 111 mg.g⁻¹ for BA based on TGA analysis. Increase of initial concentration thus allows to incorporate more CA, BA and BE in the material, BE being the most efficiently incorporated molecule. However, whereas the initial polyphenols solutions were 10 times more concentrated, the final incorporated quantity was not 10 times higher; loadings at 0.5 mM were only twice those

obtained at 0.05 mM. Moreover, no enrichment of the solid compared to the initial solution was observed as the polyphenol-to-calcium ratio in the solid was smaller than the initial solution *i.e.* 0.075 (**Table 2**).

3.2.3. Anti-oxidant properties

Anti-oxidant properties of the molecules were evaluated in solution, after adsorption on HAp NPs and after *in situ* incorporation (for the lowest initial molecule concentration). This was performed using the Blois method based on the spectrophotometric monitoring of the bleaching of a DPPH solution after 20 min of contact.^{39,40} As shown on **Figure 7**, the measured bleaching rates ranged from *ca.* 30 % to *ca.* 5 % in the following decreasing order: RA > CA > BA = BE. After adsorption, the bleaching rate range was similar while HAp nanoparticles alone had a negligible bleaching rate (< 5 %). However, the order was modified as BE > RA > BA > CA. Finally, hybrid particles formed by *in situ* incorporation all had negligible bleaching rates.

Fig. 7. DPPH bleaching rate of polyphenols in solution and after adsorption on hydroxyapatite (HAP) particles

3.3. Comparison between adsorption and *in situ* incorporation

We have assessed two methods to prepare hybrid NPs combining four natural polyphenols, rosmarinic acid, chlorogenic acid, baicalin and baicalein with hydroxyapatite, either by adsorption or *in situ* incorporation. The adsorption process occurred without significant modification of the HAp nanoparticles shape or structure, whatever the polyphenol tested. The second method allowed to obtain hybrid nanoparticles for all polyphenols, except RA when used at 0.5 mM. However, the reaction yield was decreased and calcite formation was evidenced, except in the presence of BA.

Adsorption of biologically-active molecules on HAp NPs have already been reported in the literature, for applications such as delivering antibacterial agents or osteogenic components. For instance, antibiotics such as gentamicin or tobramycin but also polyphenols, as extracts or single molecules, have been adsorbed on hydroxyapatite nanoparticles or coatings.^{36,43-48} The adsorption of bisphosphonates, such as alendronate, tiludronate or risendronate,^{24,49} on hydroxyapatite nanoparticles was described, adding osteogenic properties to the materials.

Overall, three kinds of interactions can be expected between the molecules and the surface: (1) electrostatic, (2) hydrophilic/hydrophobic and (3) complexation with the calcium ions. Electrostatic interactions are highly correlated to the surface charge of HAp: at pH below its isoelectric point (IEP), the surface is positively-charged and has good affinity for anionic species, while the mirror situation occurs at pH above IEP. The IEP of hydroxyapatite can range between 7 and 8,⁵⁰⁻⁵² so that HAp surface is positively-charged in the sorption conditions of this work. pKa of the carboxylic acid of RA, CA and BA, calculated with ACDLab Software, are 2.78, 3.90 and 2.8 respectively. RA, CA and BA are thus negatively charged in the adsorption experimental conditions (pH 5), so that attractive electrostatic interactions can drive their adsorption on the HAp surface. However, the driving force for BE adsorption cannot be electrostatic interaction since the molecule is uncharged at pH 5. Yet it is the most adsorbed

polyphenol. This result is consistent with a study by Chirdon *et al.*³² that showed that catechol and pyrogallol derivatives adsorbed more readily and strongly on HAp surfaces than phenol and carboxylic acids. The proposed explanation is based on cooperative hydrogen-bond interactions thanks to the presence of two or three close phenol groups. The fact that BA is less efficiently adsorbed than BE evidences that the loss of one available OH group on the pyrogallol ring is not compensated by the presence of an additional carboxylate group on the sugar moiety. Accordingly, the catechol ring present on CA and RA is less efficient than the pyrogallol ring of BE to interact with the HAp surface.

Following an *in situ* approach, the synthesis of hydroxyapatite in the presence of polyphenols was successfully performed for an initial polyphenol:calcium molar ratio of 0.0075. It led to hybrid nanoparticles of smaller size than in the absence of polyphenols while their crystallite size remained unchanged. In the presence of rosmarinic acid with a polyphenol:calcium molar ratio of 0.075, the formation of hydroxyapatite was inhibited while HAp NPs were obtained in the presence of CA, BA and BE in the same conditions. Such an inhibition was previously reported for other natural polyphenols, such as gallic acid and tannic acid, but the underlying mechanisms were not studied in details.^{37,38}

Electrostatic interactions leading to the formation of complexes between polyphenol and Ca^{2+} could be expected to take place when using the *in situ* method since it involves a preliminary step where $\text{Ca}(\text{OH})_2$ is dissolved in the presence of the polyphenols which are negatively charged at pH 12. Except for BE, electrostatic interactions are likely to take place between calcium ions and polyphenols through their carboxylic group. Additionally, phenolic groups are likely to be also deprotonated at pH 12 and can also interact with calcium ions. However, several authors have suggested that BA and BE are not stable in alkaline pH.⁵³ This hypothesis was confirmed by UV-Vis spectroscopy (**Fig. S4** in ESI) showing a clear decrease of the absorbance intensity in wavelength ranges representative of aromatic hydroxyl groups

for CA, BA and BE. A degradation pathway involving an oxidation process of these phenolic groups has been suggested in the literature although the final molecules could not be identified.⁵³

Besides direct interactions with calcium ions in solution, polyphenols may influence other steps of HAp formation process. To start with, they may interfere with calcium hydroxide dissolution. To check this hypothesis, the time evolution of a $\text{Ca}(\text{OH})_2$ suspension in the absence or presence of RA was followed by XRD measurements (**Fig. S5** in ESI). In absence of RA, after 15 minutes, a very low amount of solid could be recovered, suggesting fast dissolution of calcium hydroxide. Such a result is also consistent with the solubility value of calcium hydroxide in water, 1.48 g.L^{-1} at 25°C ,⁵⁴ whereas the calcium hydroxide concentration after its complete dissolution was expected to be 0.74 g.L^{-1} in the chosen experimental conditions. After 30 minutes, the solid content recovered from the solution increased and XRD indicates that it consists of pure CaCO_3 . Formation of calcite in these conditions is indeed thermodynamically favored, as previously reported in the literature.^{54,55} Similar dissolution process of $\text{Ca}(\text{OH})_2$ was observed in the presence of 0.05 mM and 0.5 mM of RA, except that in the latter case, traces of solid $\text{Ca}(\text{OH})_2$ were detected in addition to calcite. These would indicate that RA has only a minor influence on $\text{Ca}(\text{OH})_2$ dissolution.

The second possibility is that polyphenols inhibit the reaction between calcium carbonate and phosphate ions. In absence of RA or in the presence of 0.05 mM of RA, HAp was formed, while in the presence of RA at 0.5 mM only CaCO_3 was identified. Synthesis with BE, BA and CA led to intermediate situations with both CaCO_3 and HAp being present. The role of RA at 0.5 mM would therefore rather be to inhibit CaCO_3 dissolution and consequently HAp formation. One important information is that the crystallite sizes are similar for HAp and HAp-CA/BE/BA, which could result from similar nucleation conditions. On the other hand, TEM images show that all polyphenols induce a decrease in particle size, which could be a

consequence of a growth inhibition of HAp. Such a growth limitation can result either from adsorption of the polyphenols on the surface of growing particles of HAp or a depletion in available Ca^{2+} . The first possibility is very unlikely because the most efficiently adsorbed polyphenol on pre-formed HAp NPs is BE while this molecule does not show a particular inhibiting effect here. Accordingly, RA did not exhibit any specifically high affinity for HAp surface but has the highest inhibiting action on precipitation. Altogether, this would favor the hypothesis that the underlying mechanism is mainly related to polyphenol interactions with CaCO_3 that would limit its dissolution. The latter hypothesis is in line with previous reports showing that some carboxylic derivatives at low concentrations (less than 0.01 mol.L^{-1}) are able to inhibit the dissolution of CaCO_3 in alkaline pH.^{56,57} Inhibition was attributed to the blocking of dissolution sites by adsorbed carboxylates. Among the four tested polyphenols, RA, with one carboxylate and two catechol functions, may thus be the most efficiently adsorbed on CaCO_3 , preventing its dissolution. This may explain why calcite was the main mineral phase identified by XRD in the presence of high amount of this molecule.

Finally, measurements of antioxidant properties of the particles using the Blois method showed that the *in situ* approach led to inactive nanomaterials. This is in agreement with the above-suggested degradation of the polyphenols due to the harsh pH conditions of the *in situ* synthesis. However, an alternative, and possibly additional, explanation is that polyphenols are incorporated inside the nanoparticles and are therefore not accessible to DPPH. In contrast, all hybrid particles obtained by adsorption showed some antioxidant activities. It is first worth mentioning that, due to experimental constraints, the concentration of polyphenols in particle suspension is significantly larger than in the polyphenol solutions, so that bleaching rates cannot be compared between these two sets of experiments. Highest bleaching rates were obtained either for high loadings of BE with low intrinsic activity or low loadings of RA with high intrinsic activity. This strongly suggests that enhancing further the amount of adsorbed

polyphenol, for example using mesoporous nanoparticles exhibiting high specific surface area, would allow to obtain nanomaterials with strong antioxidant properties.

4. Conclusions

The feasibility of associating natural polyphenols with hydroxyapatite nanoparticles synthesized by precipitation in aqueous medium was established. The *in situ* incorporation approach was successful in most tested conditions, but the presence of organics lowered the synthesis yield or even totally inhibit hydroxyapatite formation. This could be attributed to the ability of polyphenols to inhibit the dissolution of CaCO_3 , an intermediary phase in the precipitation reaction. Moreover, obtained resulting particles showed no antioxidant activity, probably due to polyphenol degradation under selected synthetic conditions. On the contrary, post-synthesis adsorption did not modify the size of hydroxyapatite particles, leading to hybrid nanoparticles with antioxidant properties. However, polyphenols loading was limited by their poor solubility in aqueous solutions. Considering the wide variety of plant-extracted polyphenols exhibiting therapeutic properties, these results provide useful guidelines for the improved integration of natural bioactive molecules within nanobiomaterials.

Authorship contributions

Estelle Palierse: Conceptualization, Investigation, Formal analysis, Visualization, Validation, Writing - Original Draft. **Sylvie Masse:** Methodology, Investigation, Writing - Review & Editing. **Guillaume Laurent:** Methodology, Investigation, Visualization, Formal analysis, Writing - Review & Editing. **Patrick Le Griel:** Investigation, Writing - Review & Editing. **Gervaise Mosser:** Investigation, Writing - Review & Editing. **Thibaud Coradin:** Funding acquisition, Project administration, Conceptualization, Supervision, Validation, Writing -

Original Draft. **Claude Jolivald:** Funding acquisition, Project administration, Conceptualization, Supervision, Validation, Writing - Original Draft.

Conflicts of interest

There are no conflicts to declare.

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