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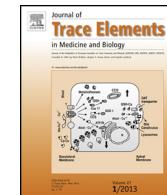
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## Pathobiochemistry

## Aluminum inhibits the growth of hydroxyapatite crystals developed on a biomimetic methacrylic polymer

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## ABSTRACT

**Project:** Aluminum (Al) is an increasing problem in biomedicine since it can interact with phosphates. Bone is one of the preferential target tissues of Al deposition: Al interacts with mineralization and/or bone cell activities. We searched the influence of Al deposition in hydroxyapatite developed on a biomimetic polymer (carboxymethylated poly(2-hydroxyethyl-methacrylate)) which mimics bone mineralization in the absence of cells.

**Procedures:** Pellets of polymer were incubated for 5 days in a synthetic body fluid (SBF) to induce mineralization, then 21 days in SBF containing 20, 40 and 60 µg/L Al<sup>3+</sup>. Other pellets were incubated in SBF containing commercial Al foil (33 mg/vial) either in 1, 2 or 6 pieces. The mineral deposits were dissolved in HCl and Ca<sup>2+</sup>, PO<sub>4</sub><sup>3-</sup> and Al<sup>3+</sup> content was measured. Hydroxyapatite was characterized by SEM and X energy-dispersive X-ray analysis (EDX).

**Results:** The amount of Al<sup>3+</sup> was dose-dependently increased in Ca/P deposits on the polymer pellets. At high concentration (or with the 6 Al foils) growth of hydroxyapatite calcospherite was inhibited; only calcified plates emerging from the polymer were observed. Pellets incubated with 1 and 2 Al foils exhibited a reduction in calcospherite diameter and an increase in the Al<sup>3+}/Ca<sup>2+</sup> ratio. EDX identified Al in the mineral deposits.</sup>

**Conclusions:** In this acellular model, Al<sup>3+</sup> altered the growth of calcospherites at low concentration and inhibited their development at high concentration. In SBF, a release of Al<sup>3+</sup> from aluminum foils also inhibited mineralization. This study emphasizes the importance of Al in bone pathology and stresses the question of its release from biomaterials.

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## Introduction

Aluminum (Al) is the most abundant metal in the terrestrial crust. It is always found combined with other elements such as oxygen, silicon, and fluorine. Aluminum compounds have many different uses, from water-treatment to abrasives and furnace linings. They are also found in consumer products such as antacids, astringents, buffered aspirin, food additives, cosmetics, antiperspirants and medical devices [1].

Although aluminum is commonly encountered in the day-to-day life, little is known about its biological roles [2]. Al can enter the body through ingestion from water or food consumption, especially

processed food with food preservatives and/or colorants containing Al [3]. Sodium-Al phosphates are considered as safe emulsifying salts in food industry [4]. Al hydroxide is commonly used to stabilize vaccines and, when injected, can create macrophage myofaciitis [5]. Estimates of Al intakes range from 0.7 mg/day for infants and can reach up to 8–9 mg/day for adults [6]. Al can also be in contact with biological tissues and ions can be released by corrosion from medical implants (e.g. hips prosthesis or dental implants made with the TA6V alloy). Al has been suspected to contribute to the appearance or development of various disorders of the nervous system, as well as of the bone and muscles in humans [2,7,8], namely dementia, anemia, myopathy, bone and joint disease [5,9–11].

Studies have confirmed that Al is absorbed through the gastrointestinal tract in healthy subjects. However, Al is not an essential trace element in the body [12] and is removed from the body after accumulation in cells of the distal kidney tubule and their desquamation in urine. When kidney functions are altered (renal insufficiency) or when intestinal absorption is increased (e.g. increased intestinal permeability [13]), Al can accumulate in two

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preferential organs: brain (linked to the phosphoproteins, lipids or phosphate groups of DNA [14]) and bone (linked to the phosphate groups of hydroxyapatite, the major Ca/P salt of the bone matrix) [15]. Several decades ago, aluminum encephalopathy associated with osteomalacia have been recognized as the major complications of chronic renal failure in dialyzed patients caused by the contamination of the dialysate by Al and the use of Al-containing phosphate binders [16,17]. When Al has been completely removed from the dialysate and the use of Al-based phosphate binders reduced, the disease has considerably declined.

Al can accumulate in bones at the mineralization front, it inhibits mineralization (leading to osteomalacia) and inhibit osteoblast activity [18–20]. However, the respective role (or entanglement of these factors) is still a matter of debate since it has been suggested that Al deposition in ostomalic bone could be a secondary phenomenon that has no influence on mineralization [21].

In this study, we have investigated the role of aluminum on the *in vitro* growth of synthetic hydroxyapatite crystals. We used a modified polymer, carboxymethylated poly(2-hydroxyethyl methacrylate) (pHEMA-CM), which mimics the calcification of woven bone in the complete absence of cells [22]. Pellets of the polymer were incubated in a synthetic body fluid containing either aluminum ion ( $\text{Al}^{3+}$ ) at varying concentrations or with pieces of commercial Al foils. The hydroxyapatite growth in the presence of Al was assessed by chemical analysis, scanning electron microscopy and energy dispersive X-ray analysis.

## Materials and methods

### The monomer

Commercial 2-hydroxyethyl methacrylate (HEMA) was purchased from Sigma-Aldrich Chemical (Illkirch, France). It is known that, due to the fabrication process, the monomer contains residual methacrylic acid, and ethyleneglycol dimethacrylate. The polymerization inhibitor, 4-methoxyphenol (added at a concentration of 350 ppm by the manufacturer) needed also to be removed. HEMA was purified and distilled under low pressure as previously described [22]. Others chemicals were purchased from Sigma-Aldrich Chemical and used without any purification.

### Preparation of polymer pellets

The polymer was prepared by bulk polymerization as described elsewhere [22]. Polymerization was carried out at 65 °C for 24 h in polypropylene wells (Delta Microscopies, Labège, France). In this way, calibrated pellets of pHEMA ( $140 \pm 5$  mg) were obtained with a good reproducibility. Carboxymethylation of the pellets was done as follows: briefly, pellets were washed with deionized water for 30 min and soaked in 0.5 M bromoacetic acid in a 2 M NaOH solution overnight at room temperature, under gentle agitation. Pellets of the carboxymethylated polymer (pHEMA-CM) were washed five times (10 min each) in deionized water and dried in an oven at 60 °C until use.

### Incubation of pellets in synthetic body fluids

A standard synthetic body fluid (SBF 1.5×) mimicking the lymph fluid was prepared by dissolving the corresponding quantities of salts  $\text{NaCl}$ ,  $\text{NaHCO}_3$ ,  $\text{KCl}$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{MgCl}_2$  ( $6\text{H}_2\text{O}$ ),  $\text{CaCl}_2$  ( $2\text{H}_2\text{O}$ ),  $\text{Na}_2\text{SO}_4$  into demineralised water at 37 °C accord to Yamada et al. [23]. The pH of this body fluid was pH 7.4, and it was achieved by using a buffer solution of tris(hydroxymethyl) aminomethane (TRIS) and hydrochloric acid (HCl). The composition (verified on a Technicon SMA analyzer) was as follows:  $\text{Na}$ : 213.28 mM;  $\text{Ca}$ : 3.73 mM;  $\text{Mg}$ : 2.25 mM;  $\text{HCO}_3$ : 6.3 mM;  $\text{Cl}$ : 212.31 mM;  $\text{HPO}_4$ :

1.35 mM;  $\text{SO}_4$ : 0.75 mM;  $\text{K}$ : 4.85 mM. Pellets of pHEMA-CM were immersed in capped Falcon tubes filled with SBF 1.5×. Pellets were let to soak in a humidified oven at 37 °C, with 5%  $\text{CO}_2$  for five days to allow the formation of hydroxyapatite globules at their surface. Then, pellets were randomly allocated in one of the following groups: (i) pellets incubated with SBF served as controls; (ii) pellets incubated with SBF enriched with 20  $\mu\text{g/L}$   $\text{Al}^{3+}$ ; (iii) pellets immersed in SBF enriched with 40  $\mu\text{g/L}$   $\text{Al}^{3+}$ ; (iv) pellets incubated with 60  $\mu\text{g/L}$   $\text{Al}^{3+}$ . The  $\text{Al}^{3+}$  ions were provided by dissolving  $\text{AlCl}_3$  in the SBF (Sigma-Aldrich). Three additional groups of pellets were incubated with freshly cut pieces of commercial Al foil (food grade, 16  $\mu\text{m}$  in thickness): (v) pellets immersed in SBF containing one piece of 60 mm × 20 mm Al foil (total mass:  $33 \pm 5$  mg); (vi) pellets incubated in SBF containing two pieces of 30 mm × 20 mm Al foil and (vii) pellets incubated in SBF containing six pieces of 20 mm × 10 mm aluminum foil. Between these three groups, the overall surface and mass of Al foil was maintain constant, only the number of pieces and as such the exposed cutting surface changed. Pellets were incubated for a 21 day-period at 37 °C in a humidified oven with an inflowing air containing 5%  $\text{CO}_2$ . The medium was replaced every two days. At the end of the incubation period, pellets were rinsed in deionized water 3 times for 10 min to remove any non-crystallized ions. Eighty-four pellets were used for the entire study and twelve pellets were allocated in each group. The following analyses were conducted in triplicate.

### Chemical analysis

Pellets were transferred into 1 mL of 0.2 M HCl for 24 h to allow complete dissolution of the mineral deposits. The fluid was then collected and used to determine the amount of free ions on an automated Hitachi 917 spectrophotometer (Roche, France) with standardized clinical reagents for calcium (Calcium Infinity TM Arsenazo III) and phosphate (the reduced phosphomolybdate method) obtained from the manufacturer. Al concentration was determined by Inductively Coupled Plasma Atomic Emission Spectrometry, ICP-AES (Jobin-Yvon JY-238 UltraTrace, HORIBA Jobin-Yvon, Longjumeau, France). Measurements were performed on the fluid obtained from three disks incubated in the same conditions, and the mean  $\pm$  SD of the triplicate was considered.

### Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX)

Pellets to be examined by SEM were processed as previously described [24]. Briefly, they were dehydrated and carbon-coated with a MED 020 device (Bal-Tec, Balzers, Liechtenstein). SEM was performed on an EVO LS10 (Carl Zeiss, Nanterre, France) microscope equipped with an energy-dispersive X-ray microanalysis machine (INCA XMAX, Oxford Instruments, Oxford, UK). In order to determine the composition of the mineral deposits, EDX was performed by point analysis. The methods explore several micrometers in thickness [25]. Based on SEM photographs, a number of 50 mineral deposits were measured for each group; the size of the Ca/P deposits was done using the ImageJ freeware (NIH, Bethesda, MD).

### Statistical analysis

Statistical analysis was done with Systat statistical software, release 13 (Systat, San José, CA). All results are expressed as mean  $\pm$  standard deviation (SD). The Kruskal-Wallis non-parametric ANOVA was used to compare the differences between the groups. Results were considered significant when  $p < 0.05$ . When the ANOVA result was significant, comparison between groups was obtained by the Conover-Inman *post hoc* test.

**Table 1**

Concentrations of Al<sup>3+</sup>, Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> in pHEMA-CM pellets incubated with 1.5× body fluid enriched with Al<sup>3+</sup> (0, 20, 40, and 60 µg/L) or aluminum foil (one piece 60 mm × 20 mm; two pieces 30 mm × 20 mm; 6 pieces 20 mm × 10 mm; thickness of 16 µm and total mass of 33 ± 5 mg).

Source of Al <sup>3+</sup>	Al <sup>3+</sup> µM/mg of pHEMA	Ca <sup>2+</sup> µM/mg of pHEMA	PO <sub>4</sub> <sup>3-</sup> µM/mg of pHEMA	Al <sup>3+</sup> /Ca <sup>2+</sup>	Ca <sup>2+</sup> /PO <sub>4</sub> <sup>3-</sup>
(i) –	0.020 ± 0.003	86.1 ± 0.3	52.0 ± 0.2	<0.001	1.66 ± 0.013
(ii) 20 µg/L	0.155 ± 0.007*	86.2 ± 20.1	52.5 ± 12.0	<0.001	1.64 ± 0.008
(iii) 40 µg/L	0.245 ± 0.046*	49.8 ± 18.0*	31.5 ± 10.1*	0.006 ± 0.001*	1.58 ± 0.030*
(iv) 60 µg/L	0.679 ± 0.261*	6.0 ± 1.6*	3.9 ± 1.2*	0.013 ± 0.011*	1.53 ± 0.036*
(v) Al foil 60 mm × 20 mm	0.052 ± 0.007*	29.3 ± 11.6*	19.6 ± 8.0*	0.001 ± 0.000*	1.49 ± 0.018*
(vi) Al foil 30 mm × 20 mm	0.043 ± 0.003*	18.9 ± 12.1*	12.6 ± 2.5*	0.007 ± 0.008*	1.51 ± 0.045*
(vii) Al foil 20 mm × 10 mm	0.042 ± 0.007*	8.0 ± 4.2*	5.3 ± 8.8*	0.013 ± 0.002*	1.50 ± 0.039*

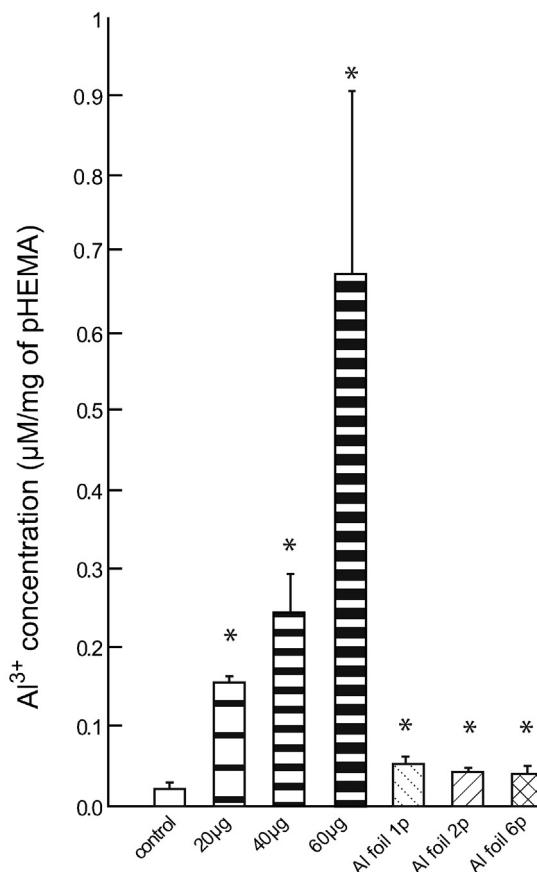
\* p < 0.05 vs. untreated.

## Results

### Chemical analysis

Pellets did not vary in shape or weight under standardized polymerization conditions.

They were translucent and their mean weight was 190 mg ± 5%, without significant differences between groups. When incubated in SBF enriched with Al ions, there were significant differences between Al ion groups and controls for chemical analysis (Table 1 and Fig. 1). As expected, the amount of Al<sup>3+</sup> was dose-dependently increased as compared with untreated controls (p < 0.0001). On the other hand, the amount of calcium and phosphorus were significantly reduced as the dose of aluminum increased. As a consequence, the Al/Ca ratio was markedly increased in a dose-dependent manner. The Ca/P ratio tended to decrease, however it did not reach significance when compared with untreated controls (Kruskal-Wallis ANOVA: 10.476, p = 0.106).



**Fig. 1.** Amount of Al<sup>3+</sup> ion in the mineral deposits deposited at the surface of pHEMA-CM pellets and dissolved in HCl. \*: significantly different from the untreated group.

The presence of Al foil pieces in the SBF also dramatically affected the chemical composition of the mineral. Indeed, a marked augmentation in aluminum concentrations and Al/Ca ratio were observed in all groups. Furthermore, the Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> content were dose-dependently reduced in these conditions as well as the Ca/P ratio.

### Scanning electron microscopy

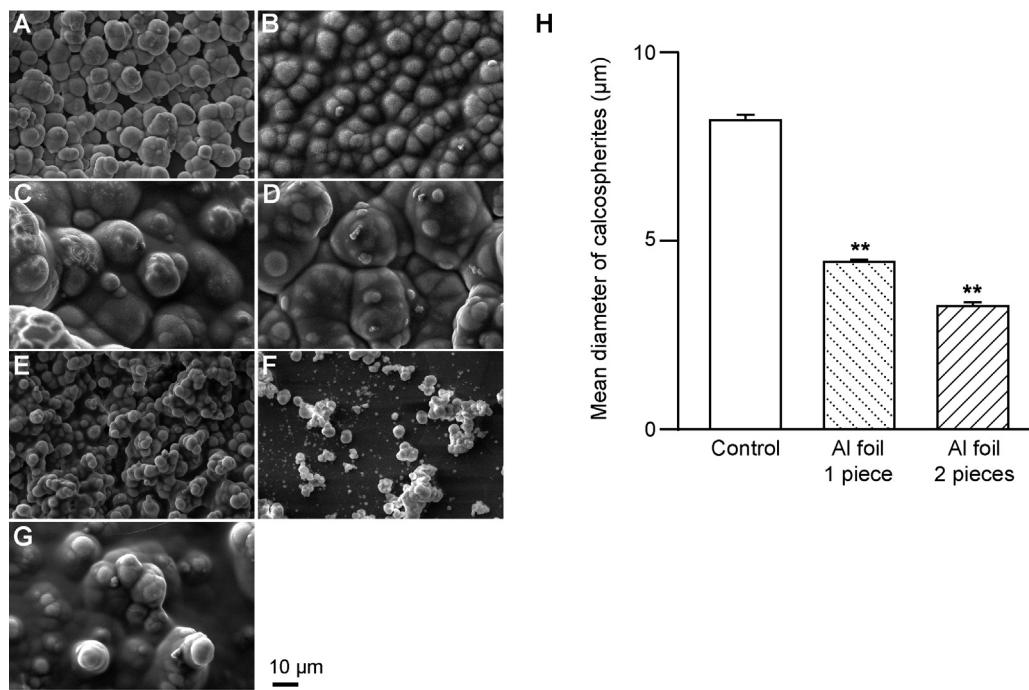
After incubation in SBF, a white layer composed of mineralized nodules was clearly seen at the surface of the pHEMA-CM pellets by SEM (Fig. 2a). Mineralized nodules had a round shape (calcospherites) made of elementary tablets or plates of hydroxyapatite, packed together with a mean diameter of 8.19 ± 0.14 µm (Fig. 2H). Significant differences were observed in the diameter and shape of mineral deposits between untreated and Al ions-treated pellets. Indeed, although a white deposit composed of calcium and phosphorus was evidenced at the surface of pHEMA disks incubated with aluminum ions, it was not composed of typical calcospherite but rather appeared as mineralized plates emerging from the polymer surface. This aspect corresponds to the nucleation areas of hydroxyapatite during the first incubation period followed by a complete arrest during the second incubation time. As such, no measurement was made possible.

Al foils were composed of pure aluminum without contaminant (Fig. 1C). Polymer disks immersed in SBF enriched with Al foil pieces also presented with a mineralized layer at their surface. With one and two pieces of aluminum foil, some calcospherites were observed at the surface of the polymer disks but with a significant reduction in their diameters as compared with controls (4.43 ± 0.07 µm and 3.26 ± 0.1 µm respectively, p < 0.001 and p < 0.001 respectively Fig. 2H). However, polymer disks incubated in SBF in the presence of six aluminum pieces presented with plate-like mineral deposits similar to those observed with the aluminum ion concentrations.

Energy dispersive X-ray analysis reveals the presence of aluminum only at the highest concentration in aluminum ion groups and on disks incubated with six pieces of aluminum foil (Fig. 3).

## Discussion

Hydroxyapatite is a critical component of the bone matrix as any alteration of the mineral lead to dramatic alteration of quality of the bone matrix and hence resistance to fracture [26]. Mineral growth on pHEMA-CM is a non-cellular model mimicking the mineralization of woven bone observed in the growing skeleton, fracture callus, and metaplasia [22,27,28]. In this system, we have repeatedly shown that mineralization is independent of any cellular machinery or protein. This system has been developed to test drug efficacy (bisphosphonates), cell adherence, and protein interaction during bone crystal growth [22,29,30]. *In vitro*, we have previously documented abnormal hydroxyapatite organization with several metals known to influence mineralization:



**Fig. 2.** Characterization of mineral deposits at the surface of pHEMA-CM after incubation with SBF 1.5×. Mineral deposits are detected in untreated controls in the form of calcospherites (A) and in disk incubated in the presence of 20 µg/L Al<sup>3+</sup> (B), 40 µg/L Al<sup>3+</sup> (C), 60 µg/L Al<sup>3+</sup> (D), 1 piece (60 mm × 20 mm) (E), 2 pieces (30 mm × 20 mm) (F) and 6 pieces of aluminum foil (10 mm × 20 mm) (G). Calcospherites are visible in A, E and F. (H) represents the mean diameter of calcospherites observed in A, E and F. \*\**p* < 0.001 vs. untreated.

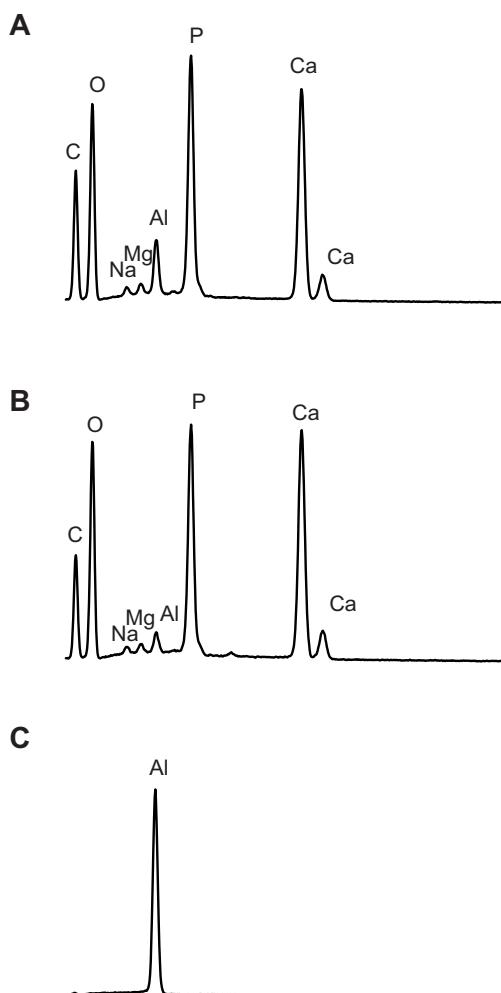
strontium [31], iron [32], cobalt, chromium and nickel [33]. In this study, AlCl<sub>3</sub> was added in a TRIS-HCl buffered solution at pH 7.4 and the majority of Al ions are complexed with hydroxyphosphate/phosphates [34]. First of all, it appeared that Al ions mediated a significant inhibition of mineral deposit at the surface of pHEMA-CM disks dose-dependently. Furthermore, as Al<sup>3+</sup> reduces mineralization, we derived the Al<sup>3+</sup>/Ca<sup>2+</sup> index to assess the incorporation of Al<sup>3+</sup> in the mineral composition. The augmentation of Al ions in SBF resulted in an increased Al incorporation into the mineral and thus led to structural modifications of the mineral deposits that did no longer appear with a calcospherite shape but rather with mineral plates emerging from the polymer surface. Because of such an intense inhibition of mineralization, it was not possible to evaluate the Al containing mineral with other methods such as X-ray diffraction or X-ray fluorescence spectrometry.

To simulate the *in vivo* release of Al ion from implanted biomaterials containing aluminum, we incubated pieces of freshly cut Al foils. This represented an easy way to bring large amount of Al ions from a material in a limited incubation period. In regenerative biomedicine, the use of TA6V (a titanium alloy containing 6% aluminum and 4% vanadium) is common to prepare orthopedic prosthesis or some types of dental implants [35]. The release of Al from TA6V has been documented *in vivo* in the microenvironment of baboons which had a segmental bone replacement with TA6V devices [36]. However, because the reduce amount of Al in this alloy, it would have necessitate a considerable incubation time to evaluate this effect by using pieces of TA6V. In addition, Ti can be released from TA6V and is also known to have deleterious effects [37]. Al release from biomaterials containing high amounts of Al have been presented: cases of Al encephalopathy have been well documented with an Al-containing cement [38] and prostheses coated with Al plasma-spray impair mineralization at their contact [39]. Extracellular fluids coming in contact with metallic biomaterials contain many anions (Cl<sup>-</sup>, PO<sub>4</sub><sup>3-</sup>, HCO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup>), cations (Na<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>), dissolved oxygen, free radicals, proteins, ...

and represent a highly corrosive environment which has a Cl<sup>-</sup> concentration equivalent to 1/3 of that of seawater and an oxygen concentration equal to 1/4 that of the air [40]. The body temperature also increases the capacity of these corrosive liquids. In addition, the SBF composition (pH, buffering, ...) frequently undergoes local fluctuations [41]. The high amounts of phosphates are very corrosive for Al which has a marked affinity for the PO<sub>4</sub><sup>3-</sup> ions [42]. Incubation of polymer disks with aluminum foils in SBF resulted in surface oxidation leading to a mineralization inhibition and Al incorporation into the mineral deposit. Noteworthy is the presence of calcospherites observed at the surface of polymer disk with one or two piece of aluminum foils. Nevertheless, incorporation of Al<sup>3+</sup> into the mineral lattice led to dramatic alterations in calcospherites size. As the possible exposed area of aluminum increased, inhibition of mineralization and mineral alterations occurred with aluminum foils.

A study in the early 80s which was conducted on 21 patients which were selected based on radiologic osteomalacia and/or suspicion of aluminum intoxication and skeletal lesions of osteitis fibrosa [28] strongly suggested that aluminum can interfere with normal mineralization. In the patient group of osteomalacic uremic bone tissue who have been exposed to aluminum-containing dialysis fluid, the preferentially localization of aluminum was at the junction of mineralized bone and osteoid tissue, where the bone mineral is normally first deposited. In rats receiving drinking water enriched with Al chloride, a decrease in the bone mineral density was observed after 150 days but no histological control of the mineralization degree was available [43]. In this study, aluminum was passively incorporated in hydroxyapatite and interfered with crystal size growth independently of any cellular, hormonal, or protein intervention. In our study, scanning electron microscopy showed a direct effect of aluminum on the crystal growth, a finding previously described by crystal modeling [44] and in synthetic hydroxyapatites [45].

The presence of metal elements in bone, such as iron, cobalt, chromium, nickel, aluminum or lead, especially in humans with



**Fig. 3.** EDX analysis of polymer disks incubated in the presence of 60 µg/L of Al<sup>3+</sup> (A) or 6 pieces of aluminum foil (B). The EDX spectrum of a native Al foil is provided in (C).

metallic implants, is well documented; however, the precise toxicity mechanisms remain largely unknown. In a human study on the effects of aluminum on bone localization, the range of Al<sup>3+</sup> found in bone was between 25 and 130 ppm [28].

One limitation of this study is linked to the acellular system used to assess the direct effect of Al<sup>3+</sup> on mineral deposition. Indeed, physiologically, bone mineralization is a complex phenomenon involving a protein pattern, deposited by bone cells that allow clear spaces for mineralization initiation and growth. The effects of aluminum on bone cells and as such protein pattern has not been investigated in the present study and represent additional mechanisms explaining the bone loss associated with Al ingestion. Recently, Al was found to induce osteoblast apoptosis which can explain the reduction of bone mass in patients with Al intoxication associated with cessation of the mineralization process [46].

## Conclusions

Al ions significantly inhibited hydroxyapatite growth at the surface of pHEMA-CM and completely modified the morphology of calcospherites, first by decreasing their mean diameter, then by converting them in less structured calcified plates. A very similar observation was reported in the same model with tiludronate, a bisphosphonic compound used as an antiosteoclastic drug. Inhibition of calcification and changing calcospherites into less-structured

plates was observed [47]. We also evidenced this severe alteration of the newly formed mineral with incorporation of Al ions into the mineral lattice by SEM and EDX. Al interfered with mineralization either when provided in the SBF or it can be released from the foils although the metal is passivated by a layer of aluminum oxide.

## Conflict of interest

None disclosed.

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