Bisphosphonate complexation and calcium doping in silica xerogels as a combined strategy for local and controlled release of active platinum antitumor compounds

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The production of bone substitute biomimetic materials which could also act as antitumoral drug release agents is of enormous interest. We report in this paper the synthesis and characterization of a novel platinum dinuclear complex containing a geminal bisphosphonate and its embodiment into xerogels prepared by the sol–gel method. Our goal was to obtain a hybrid inorganic matrix that could release a platinum species active against bone tumors or metastases, upon local implant. Two silica xerogels were considered: one was composed of pure silica, while the other contained also some calcium as potential release-modulating agent thanks to its high affinity for bisphophonates. The platinum-complex loading capacity of the inorganic matrices, the release kinetics in buffer simulating physiological conditions, and the stability upon storage were investigated as a function of Pt-complex concentration and calcium addition. We found that the presence of calcium in the composites deeply influences not only the stability of the formulations but also the nature of the platinum complex liberated in solution.

Introduction

Nowadays cisplatin is one of the five most used agents against solid tumors such as testicular, ovarian, and bladder carcinomas.[1] However, the use of cisplatin is limited by some serious drawbacks such as nausea, vomiting, ototoxicity, myelotoxicity and concentration-dependent nephrotoxicity.[2,3] Moreover it shows good activity towards a limited spectrum of cancerogenic cells.

New platinum-based antitumor agents are being developed in order to overcome the severe side-effects, to improve clinical effectiveness, and to extend the spectrum of activity. A promising strategy makes use of carrier ligands able to promote the specific accumulation of the drug in target organs or cells.[4] In this context, platinum complexes with (aminoalkyl)phosphonic acids, firstly synthesized and characterized by Appleton and colleagues,[5,6] were exploited by the group of Keppler for their potential use as anticancer drugs selective for bone tumors.[7,8] This prospect was justified by the clinical use of geminal bisphosphonates (such as Etidronate, Clodronate, Pamidronate, and Alendronate) in the treatment of hypercalcaemia and other bone related malignancies.[9–12] Pharmacological studies performed on platinum complexes with amino-bis/tris(methylenephosphonate) ligands (ATMP and BPMAA in Chart 1) proved that these multifunctional molecules have a therapeutic activity superior to that of cisplatin in an orthotopically transplanted rat osteosarcoma model which closely resembles the human osteosarcoma.[13,14]

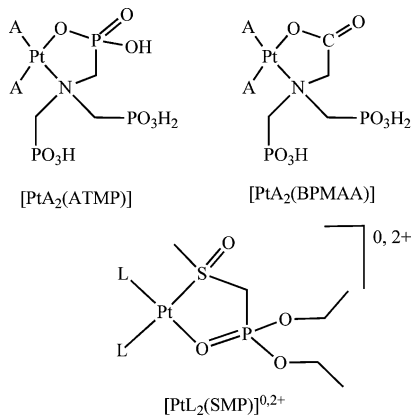

Chart 1 Sketches of platinum complexes with aminotris(methylenephosphonate) (ATMP), bis(phosphonomethyl)aminoacetate (BPMAA), and diethyl [(methylsulfinyl)methyl]phosphonate (SMP). A_2 = two amines or a chelated diamine. L_2 = two chlorido, a dicarboxylate, or a chelated diamine ligand(s).

In test animals the compounds containing bis(phosphonomethyl)aminoacetate (BPMAA) appear to have a lower spectrum of toxicity and an higher therapeutic index and to be better tolerated even in high doses than those containing aminotris(methylenephosphonate) (ATMP).[15] These compounds react with (di)nucleotides releasing the aminophosphonic moiety.[16]

In order to limit the reactivity of Keppler's compounds towards (di)nucleotides we have recently proposed the use of a chelated sulfinyl-phosphonate ligand (SMP in Chart 1), which is capable

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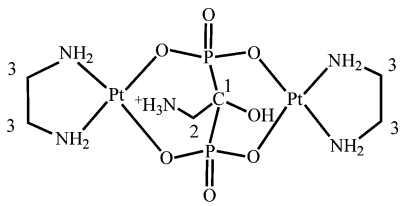
of binding rather strongly to platinum through the sulfur atom of the sulfoxide and to give chelation through the non-alkylated phosphorus-bound oxygen atom.[17,18]

We also reasoned that the affinity of bisphosphonate moieties for calcium ions may provide another means for bone localization of platinum-bisphosphonate complexes. For example, platinum-bisphosphonate complexes could be loaded onto calcium-containing matrices to be used as bone fillers. Such composites could be administered locally at the site of an osteosarcoma and act both as bone substitutes and as local drug-releasing systems with the final goal of inhibiting locally the tumor re-growth and reducing the systemic toxicity.

Towards this end, we have recently investigated the adsorption and desorption of cisplatin, alendronate, and a platinum complex containing deprotonated methylenebis(phosphonic acid) (medronate) towards two bio-mimetic synthetic hydroxyapatite nanocrystal materials having different crystal shape and chemico-physical properties.[19] The results showed a high loading capacity of the synthetic hydroxyapatite for the bisphosphonate platinum complex and the ability of the composite material to release a bisphosphonate free Pt compound to the surrounding medium in a slow and controlled manner.

In addition to hydroxyapatite, also nanoporous xerogels can be used as implantable biodegradable and bioerodable delivery systems. In particular, silica based bioactive xerogels are known to be biocompatible and slowly bioerodable after implantation[20–22] and have already been exploited for the entrapment and slow release of cisplatin.[23,24] Sol–gel silica is also known to be potentially bioactive in the sense that surface hydroxyapatite deposition can occur upon its immersion into a simulating physiological buffer. The bioactivity of sol–gel silica polymers depends upon the gel properties but is independent from the presence of calcium in the polymer composition, this was not the case for fused bioactive glasses originally developed by Hench.[25]

In this paper we describe the synthesis of a novel platinum complex specifically designed to contain a geminal bisphosphonate (Chart 2) and its loading into a silica-xerogel with the final aim of obtaining a hybrid inorganic matrix that might be used for treatment of bone tumors upon local implant.[26]



$[Pt_2(en)_2(AHBP\text{-}H)]^+$ (**3**)

Chart 2 Sketch of the novel compound with some atom numbering.

Material and methods

Chemicals

Ethylenediamine, *N*-phthaloylglycine, tris(trimethylsilyl)phosphite, tetraethoxysilane (TEOS), tetrahydrofuran (THF), and $Ba(OH)_2$ were purchased from Sigma-Aldrich (Milan, Italy). Commercial reagent grade chemicals and solvents (Sigma-Aldrich) were used without further purification.

Instrumental measurements

UV spectra were measured using a Cary m50-Scan UV-Vis spectrophotometer. Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES) measurements were performed using a Perkin Elmer Optima 4200 DV spectrometer. NMR spectra were recorded on a Bruker Avance DPX instrument operating at 300 MHz (^{1}H). Standard pulse sequences were used for ^{1}H, $^{31}P\{^1H\}$ (121.5 MHz), and $^{195}Pt\{^1H\}$ (64.5 MHz) 1D spectra. Chemical shifts are given in ppm. ^{1}H chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate (TSP). ^{31}P chemical shifts were referenced to external H_3PO_4 (85% w/w). ^{195}Pt chemical shifts were referenced to external $K_2[PtCl_4]$ in D_2O fixed at −1628 ppm. Elemental analyses were carried out with a Hewlett Packard 185 C, H, and N analyzer. A Crison Micro-pH meter Model 2002 equipped with Crison micro-combination electrodes (5 and 3 mm diameter) and calibrated with Crison standard buffer solutions at pH 2.00, 4.01, 7.02, and 9.26 was used for pH measurements. The pH readings from the pH meter for D_2O solutions are indicated as pH* values and are uncorrected for the effect of deuterium on glass electrodes.[27]

Synthesis of $[PtCl_2(en)]$ and $[Pt(en)(H_2O)(OSO_3)]$ (en = ethylenediamine). These complexes were prepared as already reported in the literature.[28]

Synthesis of 1-hydroxy-2-(*N*-phthaloylamino)ethane-1,1-diyl-bisphosphonic acid (1). *N*-Phthaloylglycine (1.5 g, 7.31 mmol) was dissolved in $SOCl_2$ (24 mL) and the resulting solution was kept under reflux for 2 h. The solvent was then evaporated to dryness under reduced pressure and the obtained pale yellow solid was suspended in THF (12 mL). This suspension was treated at 0 °C with tris(trimethylsilyl)phosphite (7.33 mL, 21.93 mmol) for 5 min, then the mixture was stirred at room temperature for 10 min and, subsequently, methanol was added to the solution. The resulting mixture was kept under stirring for 1 h at room temperature and then the volatile components were evaporated under vacuum affording a yellow oil. This latter was treated several times with diethyl ether until obtainment of a white solid, which was isolated by filtration of the solution, abundantly washed with diethyl ether, and finally dried under vacuum, affording 2.34 g of compound **1** (*ca.* 91% yield). ^{1}H-NMR (D_2O, pH* = 2.5): 7.92–7.82 (4H, m, phenyl protons) and 4.36–4.30 (2H, m, CH_2) ppm. $^{31}P\{^1H\}$-NMR (D_2O, pH* = 2.5): 17.08 ppm.

Synthesis of 2-amino-1-hydroxyethane-1,1-diyl-bisphosphonic acid (AHBP-H$_4$, 2). Compound **1** (2.34 g, 6.65 mmol) was dissolved in concentrated HCl (24 mL) and the resulting mixture was kept under reflux overnight. The solvent was then removed under reduced pressure and the brown solid suspended in ethanol (40 mL) and kept at 70 °C (water bath) for 1 h. The solid was then isolated by filtration of the solution, washed with warm ethanol and with diethyl ether, and finally dried under vacuum. The pure product was obtained by crystallization from water. Obtained 1.29 g (88% yield). Anal.: Calculated for $C_2H_9NO_7P_2$: C, 10.87; H, 4.10; N, 6.34. Found: C, 10.71; H, 4.06; N, 6.19%. ^{1}H-NMR

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(D_2O, pH* = 3.5): 3.45 (2H, t, $^3J_{H\text{-}P}$ = 11.81 Hz, CH_2) ppm. $^{31}P\{^1H\}$-NMR (D_2O, pH* = 3.5): 16.46 ppm.

Synthesis of $[Pt_2(en)_2(AHBP\text{-}H)](HSO_4)$ (3). Compound **2** (124 mg, 0.561 mmol) was dissolved in H_2O (10 mL) and the resulting solution was treated with $Ba(OH)_2{\cdot}8H_2O$ (177 mg, 0.561 mmol). The obtained white suspension was treated with a solution of $[Pt(en)(H_2O)(OSO_3)]$ (423 mg, 1.146 mmol in 10 mL of water) and the resulting mixture kept under stirring at 0 °C for 3 h and then left standing at 4 °C overnight. After removal of the white solid ($BaSO_4$), the filtered solution (pH *ca.* 3.0) was treated with additional $Ba(OH)_2{\cdot}8H_2O$ until the pH reached a value of 5.0 (required 30.3 mg, 0.096 mmol). The newly formed white solid was removed by filtration of the solution and this latter taken to dryness by evaporation of the solvent under vacuum. The yellow crystalline residue was dissolved in H_2O (1.5 mL) and the pH was lowered to 1.0 by addition of H_2SO_4 (0.5 M). The resulting acidic solution was treated with methanol which caused the precipitation of the desired product having HSO_4^- as counter-ion. The precipitate was isolated by filtration of the solution, washed with methanol and diethyl ether, and finally dried under vacuum. Obtained 329 mg (80.6% yield). Anal.: Calculated for $[Pt_2(en)_2(AHBP\text{-}H)](HSO_4){\cdot}5H_2O$ ($C_6H_{32}N_5O_{16}P_2Pt_2S$): C, 7.88; H, 3.53; N, 7.66. Found: C, 7.94; H, 3.12; N, 7.35%. ^{1}H-NMR (D_2O, pH* = 3.5): 5.93 (4H, br, ethylenediamine NH_2), 5.43 (4H, br, ethylenediamine NH_2), 3.38-3.31 (2H, dd, $^3J_{H\text{-}P}$ = 10.07 and 10.30 Hz, C(2)H_2), 2.62 (8H, br, C(3)H_2) ppm. $^{31}P\{^1H\}$-NMR (D_2O, pH* = 3.5): 37.60 ppm.

NMR experiments at different pH and calculation of pK_a values

Samples containing *ca.* 0.0016 mmol of the compound under investigation (AHBP-H_4 or $[Pt_2(en)_2(AHBP\text{-}H)](HSO_4)$) dissolved in 0.7 mL of D_2O were transferred into an NMR tube. The pH* of the samples was adjusted to the required values by addition of 0.9 M D_2SO_4 and 2.0 or 0.5 M NaOD solutions and the pH* value was measured by using a 3 mm diameter electrode for NMR tube. No control of the ionic strength was performed. Portions of the pH titration curve were fitted separately to the Henderson–Hasselbalch equation using the program *KaleidaGraph*.[29]

Preparation of Pt(II) complex-loaded silica xerogels

Two xerogel formulations, differing in the presence or absence of calcium (Ca(+) and Ca(−), respectively), were considered. Each formulation was loaded with two different amounts of Pt(II) complex (Table 1). Complex-free formulations were also used for control.

The gels were synthesized by a two-step catalyzed procedure. Initially, two pre-hydrolyzed sols were prepared by mixing tetraethoxysilane (TEOS, 1 equiv), ethanol (0.5 equiv), HCl (0.01 equiv), and water (2 equiv) which could be either pure or containing $Ca(NO_3)_2$ (0.33 equiv). Pre-hydrolysis was catalyzed by 2 h sonication in an ice bath in the case of the sol containing calcium and by 2 h heating at reflux in the case of the calcium-free sample. In a second phase, the pre-activated sols were mixed with different amounts of doubly distilled H_2O, an aqueous solution of the Pt(II) complex (45 mg mL^{-1}), and a solution of HF (2.5 M) as a catalyst for gelation. The final H_2O : Si ratio (R) in all samples was 4. Gel formation occurred within 3.5 h independently from the presence of the platinum complex. Wet gels were aged in a closed vial for three days and, after removal of the liquid expelled upon syneresis, they were dried at 50 °C until constant weight. Xerogels were manually crushed in a mortar and sieved to obtain granules with homogeneous size distribution ($\varnothing = 370 \pm 180$ microns).

Determination of platinum content

To determine the amount of complex embedded in the final formulations, weighted amounts of each xerogel were digested by treatment with 10% HF and the Pt concentration in the digested mixtures was determined by ICP-OES analysis. The mole composition of the final xerogels is reported in Table 1. For ICP analysis, the HF digested samples were diluted up to a final 0.25% acid concentration and then nebulized using a compact nebulizer having a small diameter capillary (Teflon) and a polyvinylidine difluoride (PVDF) body to minimize undesired large drop formation and improve HF tolerance. Four emission lines were used for the determination of platinum. Solution concentrations were obtained as an average of two records.

Complex release, erosion, and stability of the formulations

Release of $[Pt_2(en)_2(AHBP\text{-}H)]^+$ was investigated by immersing the xerogel granules (200 mg) into Tris buffer (100 mL of 10 mM trishydroxymethylaminomethane, 150 mM NaCl, pH 7.4) at 37 °C, under constant stirring. At scheduled times, small fractions (2 mL) of the solution were removed for Pt quantification and replaced by fresh buffer. Pt concentration was determined by ICP OES analysis. For this purpose, the samples were treated with ultrapure nitric acid in order to obtain a metal concentration between 1 and 8 ppm in 1% nitric acid, and analysed as described above. The reported concentrations are the average of two determinations. A separate set of experiments was carried out to determine whether different Pt species were released in the different phases of the process. For this purpose, the xerogels were immersed in the buffer solution as described above and, at scheduled times (1.5 and 20 h), the receiving medium was completely removed (and replaced by fresh buffer) and analyzed by UV-Vis spectrophotometry. The

Table 1 Mole ratios of silica sol formulations and amount of loaded platinum complex

Formulation	Wet gel mole composition[a]	Matrix mole composition[b]	Pt(II) complex/mg g^{-1} xerogel
Ca(−)1	$1 : 0 : 4 : 0.025 : 1.5 \times 10^{-4}$	1 : 0 : 0.025	1.9
Ca(−)2	$1 : 0 : 4 : 0.025 : 2.9 \times 10^{-4}$	1 : 0 : 0.025	2.98
Ca(+)1	$1 : 0.33 : 4 : 0.025 : 1.6 \times 10^{-4}$	1 : 0.33 : 0.025	1.24
Ca(+)2	$1 : 0.33 : 4 : 0.025 : 3.14 \times 10^{-4}$	1 : 0.33 : 0.025	1.57

[a] Si: Ca: H_2O: F^-: Pt(II)-complex mole ratio. [b] SiO_2: CaO: F^- mole ratio.

spectra of the release samples were compared with those of $[Pt_2(en)_2(AHBP\text{-}H)](HSO_4)$ and of $[PtCl_2(en)]$.

Matrix erosion was analyzed by measuring the amount of silicic acid released in the medium using the molybdenum-blue colorimetric test.[26] Release and erosion tests were performed one and thirty days after xerogel preparation to verify the stability of the formulations upon storage (in closed vials and at room temperature). Release and erosion data were mathematically elaborated and were plotted as $(M_t/M_{tot})\%$ *vs* time, where M_t is the amount of total compound released at time t, and M_{tot} is the total compound contained in the sample.

Results and discussion

Synthesis and characterization of the platinum complex

The synthesis of the ligand (2-amino-1-hydroxyethane-1,1-diyl)bisphosphonic acid (AHBP-H_4, **2**) has been carried out by a mixture of two different published procedures (Scheme 1).[30,31]

Initially a NH_2-protected glycine (*N*-phthaloylglycine) was converted into the corresponding acyl chloride and used as substrate in a modified Arbuzov reaction in which tris(trimethylsilyl)phosphite was used as phosphorylating agent. The reaction led to the silicic ester of the geminal bisphosphonic acid, which was hydrolyzed by treatment with methanol to give compound **1**. After evaporation of the volatile fractions, deprotection of the aminic group was achieved by treatment with concentrated HCl. This treatment led to formation of **2** and phthalic acid which was removed by washing with hot absolute ethanol.

The characterization of **2** (present in the zwitterionic form) has been performed *via* elemental analysis and ^1H and ^{31}P-NMR spectroscopy in D_2O at pH* = 3.5. The triplet at 3.45 ppm in the ^1H-NMR spectrum was assigned to the methylene protons. The splitting of the methylene signal in a triplet is caused by coupling with two equivalent phosphorus atoms ($^3J_{H,P} = 11.81$ Hz). The $^{31}P\{^1H\}$ NMR (pH* = 3.5) showed a singlet at 16.46 ppm assigned to the two P atoms.

The synthesis of $[Pt_2(en)_2(AHBP\text{-}H)](HSO_4)$ (**3**) was performed starting from the barium salt of **2** (BaAHBP-H_2) and $[Pt(en)(H_2O)(OSO_3)]$. Initially, the reaction was carried on by mixing the reactants in a 1 : 1 molar ratio (the aim was to prepare the 1 : 1 adduct), however, under these conditions, we obtained a mixture of two products: a major product corresponding to the dinuclear species described in this work and a minor product corresponding to a mononuclear species having one bisphosphonate molecule per Pt(en) moiety. Unfortunately the two products are extremely difficult to separate. Therefore we repeated the reaction using two equivalents of $[Pt(en)(H_2O)(OSO_3)]$ for one equivalent of barium bisphosphonate so as to favor the formation of the dinuclear species.[32,33] The elemental analysis of the resulting compound was in accordance with the presence of one aminobisphosphonate and two Pt(en) residues (**3**, Chart 2). The characterization of **3** has been performed *via* ^1H, ^{31}P, and ^{195}Pt-NMR in D_2O. The ^1H-NMR spectrum (Fig. 1) shows two broad singlets at 5.93 and 5.43 ppm, assigned to the aminic protons of coordinated en (geminal protons of each aminic group are made inequivalent by the lack of symmetry with respect to the coordination plane).[34,35] The acidity conditions slow down the exchange of the aminic protons with the deuterium of the solvent, allowing detection of the aminic protons in water solution. The doublet of doublets centered at 3.34 ppm was assigned to the protons of the methylene group of the bisphosphonate (position 2 in Chart 2). These protons are shifted at higher field ($\Delta\delta = -0.11$ ppm) as compared to the free ligand. The two methylenic protons are magnetically equivalent, however each of them has

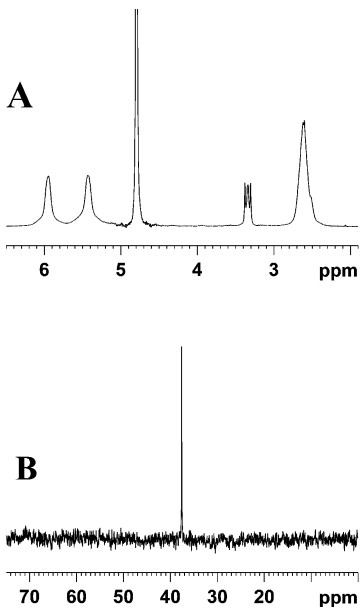
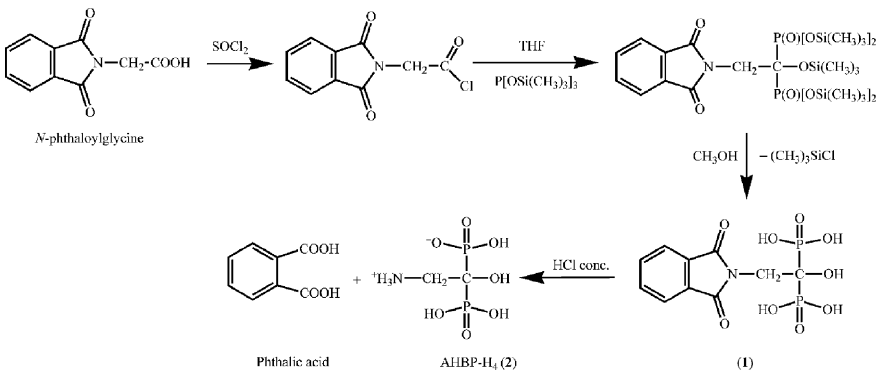


Fig. 1 ^1H (A) and ^{31}P (B) NMR spectra of compound **3** in D_2O.



Scheme 1

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different coupling constants with the two phosphorus atoms ($^3J_{H-P}$ = 10.07 and 10.30 Hz) resulting in a doublet of doublets. Finally, the broad signal at 2.62 ppm was assigned to the methylene protons of the Pt(en) residues.

The ^{31}P-NMR spectrum (Fig. 1) of $[Pt_2(en)_2(AHBP\text{-}H)]^+$ in D_2O at pH* = 3.5 shows a singlet with unresolved platinum satellites (platinum satellites usually broaden with an increase in molecular size and chemical shift anisotropy)[35] falling at 37.60 ppm and assigned to the two phosphorus atoms of the phosphonic groups, which are magnetically equivalent and shifted at lower field ($\Delta\delta$ = 21 ppm) with respect to the free AHBP ligand at the same pH*. Such a lower field shift of the phosphorus nuclei is characteristic of a phosphorus atom included in a 5-membered ring,[6,36] however in **3** the P atoms are included in 6-membered rings, therefore the downfield shift likely depends upon electronic rather than geometric effects.

The ^{195}Pt-NMR spectrum (Fig. 2) shows two broad signals at −1691 and −1707 ppm. These signals are at lower field with respect to the starting $[Pt(en)(H_2O)_2]^{2+}$ substrate (−1922 ppm). The same trend has already been observed in platinum complexes with a diamine and a dicarboxylate ligand with respect to the species with a diamine and two aqua ligands.[37–39]

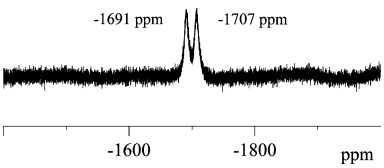


Fig. 2 ^{195}Pt NMR of **3** in D_2O.

The presence of only one signal in the ^{31}P spectrum and of two signals in the ^{195}Pt spectrum strongly supports a structure in which one bisphosphonate unit bridges two platinum atoms (W conformation).[33] The two phosphorus atoms are chemically equivalent because of the presence of a plane of symmetry passing through the two platinum atoms and perpendicular to the coordination plane. In contrast, the absence of a plane of symmetry passing through the P–C(1)–P atoms and perpendicular to the coordination plane (presence of two different substituents, –OH and $–CH_2–NH_3^+$, on the C(1) carbon atom), renders the two platinum atoms inequivalent. The protonated amino group is expected to exert a deshielding effect on the nearby Pt atom, therefore the less shielded platinum signal (−1691 ppm) is assigned to the platinum atom facing the protonated aminomethyl group. Our data exclude the involvement of a deprotonated hydroxylic group in coordination to platinum as observed in the complexes of many other metals with tricoordinate bisphosphonates.[40]

Determination of pK_a's *via* ^{31}P-NMR pH titrations

For a full characterization of the new compounds we performed ^{31}P-NMR experiments at different pH on the free aminobisphosphonate ligand and on the corresponding platinum complex.

Acid dissociation constants have been previously reported for alendronate (4-amino-1-hydroxybutane-1,1-diyl-bisphosphonic acid),[33] which is a commercial drug having a structure very similar to that of the aminobisphosphonate used in this study. Alendronate exhibits five protonation constants spread over the whole range of pH. The plot showing the change in ^{31}P chemical shift as a function of pH in the case of free AHBP is given as Fig. 3. The titration curve shows changes in the slope (inflexion points) corresponding to the various deprotonation steps (Scheme 2). We did not determine the constant related to the first deprotonation step (pK_{a1} in Scheme 2) because this requires ionic hydrogen concentrations beyond the reach of our experimental conditions.

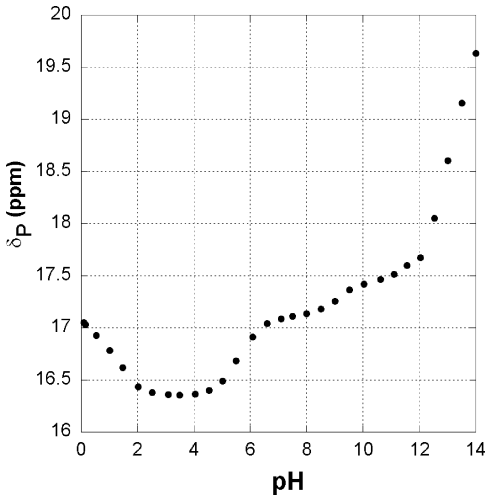
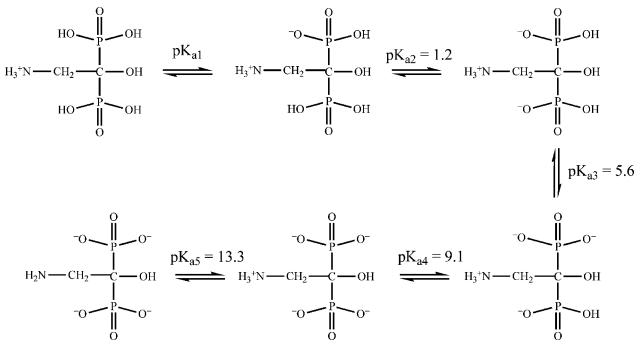


Fig. 3 Plot of ^{31}P chemical shift *vs* pH* for **2**.



Scheme 2

As the pH* of the sample was increased from *ca.* 0 to 3 we observed a shift to higher field of the ^{31}P chemical shift with an inflexion point (calculated by fitting the points between pH* 0 and 3 in Fig. 3 to the Henderson–Hasselbalch equation) falling at pH* 1.12. This first constant corresponds to the second deprotonation step (pK_{a2}, Scheme 2) of the AHBP ligand. Deprotonation of a phosphonate OH is known to shield the phosphorus nucleus directly attached to it and to deshield the distant second phosphonate group present in the molecule. The former effect is called the "direct effect" while the latter effect is called the "remote effect," both effects were described very accurately by Appleton in the pH titrations performed on the BPMAA ligand (Chart 1).[6] We propose that these effects apply also to the present case and that for the second deprotonation (pK_{a2}) the direct effect prevails over the remote effect. The global result is a shielding of the phosphorus nuclei.

By further increasing the pH* from 3 to 7 we observed a deshielding of the phosphorus nuclei with an inflexion point falling

at pH* 5.61. This deprotonation step corresponds to bisdeprotonation of only one phosphonate group (pK_{a3} in Scheme 2). The deshielding of the ^{31}P nuclei indicates that in this deprotonation step the remote effect prevails over the direct effect.

By further increasing the pH* from 7 to 14 we observed a further deshielding of the phosphorus signal with two inflexion points: one falling at pH* 9.23 (pK_{a4}) and the other at pH* 13.14 (pK_{a5}). The former deprotonation step (pK_{a4}) most likely corresponds to the bisdeprotonation of both phosphonate groups. Once again the remote effect is higher than the direct effect, but the difference is not large so that only a small global deshielding of the phosphorus atoms is observed. The last inflexion point (pK_{a5}) is characterized by a huge deshielding of the phosphorus nuclei and is typical for the deprotonation of the ammonium group in an aminoalkylphosphonate species.[41] The great deshielding is caused by the loss of the zwitterionic form in which the positively charged ammonium moiety is electrostatically attracted by the (partially or fully) deprotonated phosphonate groups and in this conformation causes a shielding of the phosphorus atoms.[42] Therefore our data are in full agreement with the data obtained for analogous aminobisphosphonates.[43]

The pH titration was also performed on a sample of the platinum complex **3**. Differently from the free aminobisphosphonate ligand, a constant ^{31}P chemical shift was observed indicating that this is not influenced by pH* (pH* range 0–8). This result supports the involvement of all phosphonate hydroxyl oxygens in coordination to platinum. The platinum-coordinated oxygen atoms cannot undergo either protonation or deprotonation steps as the pH* is changed and therefore there are no breaks in the curve of δ_P against pH. Unfortunately, compound **3** undergoes structural changes at pH* higher than 8.0 with the formation of new product(s) having inequivalent phosphorus atoms. Most probably the deprotonated aminic group coordinates to platinum displacing one oxygen atom of a bis-coordinated phosphonate. The resulting product with a bis-coordinated and a monocoordinated phosphonate group would have inequivalent phosphorus atoms. No release of free AHBP was observed, indicating that a rearrangement and not a decomposition of the dinuclear platinum complex occurs at pH* > 8.

Pt-complex release from silica xerogels, matrix erosion, and stability of the formulations

The Pt–bisphosphonate complex was loaded onto two xerogel formulations differing in the presence or absence of calcium in their compositions. The rationale for introducing calcium into the gel was twofold: (i) to improve its bioactivity and (ii) to increase its affinity for bisphosphonic moieties. In particular, the presence of calcium in silica polymers is known to improve their bioactivity[44,45,25] by promoting hydroxyapatite (HA) deposition on their surface, which favors implant grafting to the surrounding bone tissue. Therefore calcium containing xerogel formulations known from the literature to be bioactive were selected for this work.[20,22] Moreover, since bisphosphonic moieties have high affinity for calcium ions, the presence of calcium could influence significantly also the loading and the release processes. Each formulation (Ca(+) and Ca(−)) was loaded with two different amounts of Pt complex so as to measure the loading capacity of each formulation.

The four samples were analyzed for platinum release the day after xerogel preparation and one month later (Fig. 4). Moreover, simultaneously with the platinum release, was also investigated the matrix erosion (Fig. 5). The results thus provide information regarding the effect of calcium and of Pt complex concentration on (i) matrix loading capacity, (ii) rate of release, and (iii) stability upon long term storage.

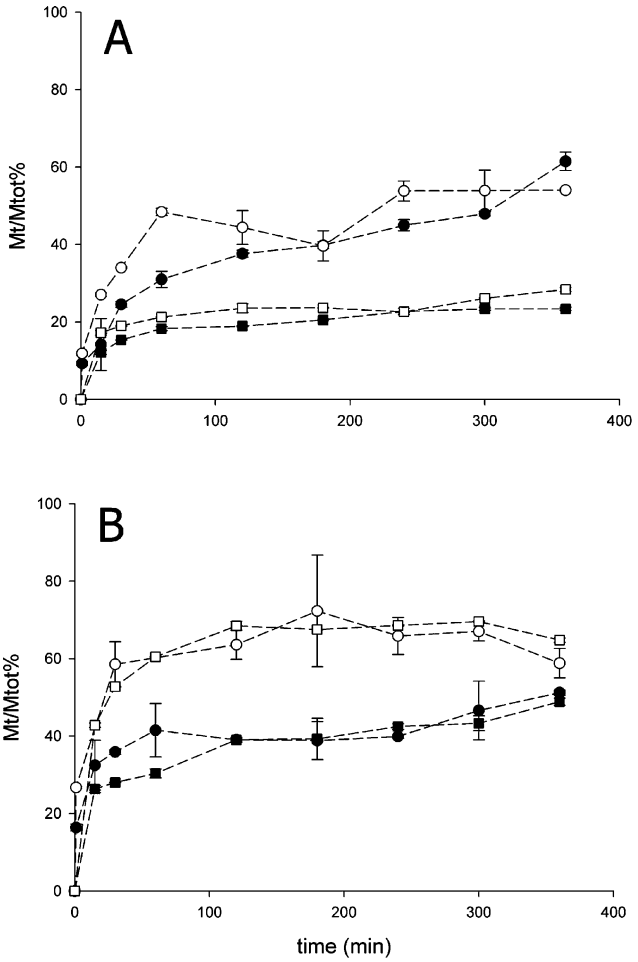


Fig. 4 Kinetics of Pt complex release from (A) Ca(−) and (B) Ca(+) formulations containing Pt : Si ratio of *ca.* 1.5×10^{-4} (1, full symbols) and 3.0×10^{-4} (2, open symbols) as measured one day after xerogel preparation (d1, circles) and 30 days after preparation (d30, squares). A: (●) Ca(−)1-d1; (■) Ca(−)1-d30; (○) Ca(−)2-d1; (□) Ca(−)2-d30. B: (●) Ca(+)1-d1; (■) Ca(+)1-d30; (○) Ca(+)2-d1; (□) Ca(+)2-d30.

All release profiles were characterized by an initial burst (occurring within the first 60 min) followed by a slow and long lasting Pt-release process. The amount of complex released in the initial burst varied from 20 to 60% of the loaded compound, depending upon the formulation under investigation and the time of storage before testing. It is likely that the portion of complex that is released during the initial burst is located at the granule surface and is released by a dissolution process. In contrast, the portion of complex that is released in the slow phase is embedded in the polymer matrix. The amount of complex embedded in each formulation was thus calculated by subtracting the fraction released in the initial burst from the total amount used in the

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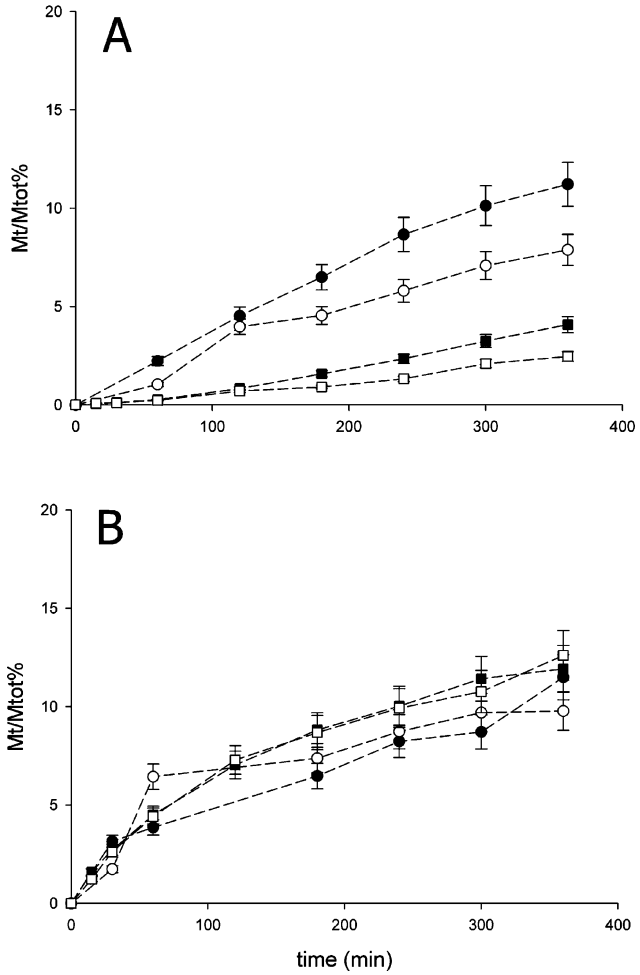

Fig. 5 Silica erosion from (A) Ca(−) and (B) Ca(+) xerogels containing Pt : Si ratio of *ca.* 1.5×10^{-4} (1, full symbols) and 3.0×10^{-4} (2, open symbols) analyzed one day after their preparation (d1, circles) and 30 days later (d30, squares). A: (●) Ca(−)1-d1; (■) Ca(−)1-d30; (○) Ca(−)2-d1; (□) Ca(−)2-d30. B) (●) Ca(+)1-d1; (■) Ca(+)1-d30; (○) Ca(+)2-d1; (□) Ca(+)2-d30.

xerogel preparation (Table 2). The concentration of complex in each matrix (Pt mg g^{-1} xerogel) was thus calculated and was defined as the 'loading capacity' of that formulation.

The results indicate that the presence of calcium reduces the matrix loading capacity while increasing the amount of platinum compound released in the initial burst. Moreover the calcium containing formulations appear to have a maximum loading capacity (*ca.* 0.6–0.8 mg complex g^{-1} xerogel) which does not depend upon the initial drug load. Finally, the release behavior of the Ca(+) samples and their erosion profile do not change upon storage for 30 days, thus indicating that these samples are stable with time and therefore suitable for practical application. In contrast, the Ca(−) samples display a different behavior: the loading capacity appears to be higher and to depend upon the initial platinum concentration and the aging of the sample. Moreover the release behaviour and the erosion kinetics also change upon aging indicating that such formulations are rather unstable.

In order to better understand the mechanism underlying the release process, the slow Pt-release was compared with the erosion process. In order to allow also the Ca(−) sample to reach stability, the investigation was performed only on samples stored for 30 days at room temperature. In both cases (Ca(+) and Ca(−) samples) the platinum release and the matrix erosion data, plotted against the time square root (data not shown), were linear, indicating that both phenomena depend upon a diffusion process, in agreement with previous observations.[46] The times required for total matrix erosion and Pt release (time of exhaustion, *t*-ex) were estimated from the slopes of the linear plots and are reported in Table 3. The data indicate that platinum release is faster than matrix erosion for both Ca(+) and Ca(−) formulations and that both erosion and diffusion were significantly faster in the presence of calcium.

The different Pt-complex loading capability demonstrated by the Ca(+) and Ca(−) formulations probably reflects structural differences between the two xerogels. Indeed, the calcium content in the Ca(+) formulation was quite high (Ca : Si = 0.33 : 1 mol : mol, Table 1) and could have a significant impact on the inner structure of the polymer network resulting in a reduced polymer "solubility" of the platinum compound. The latter structural feature could be totally unrelated to the chemical affinity of calcium ions for the bisphosphonic moiety of the platinum complex, which, instead, can deeply affect the release behaviour.

Table 3 Estimated time required for total Pt-complex release (*t*-ex Pt-release) and silica erosion (*t*-ex Silica erosion) performed on aged samples (30 days storage)

Formulation	*t*-ex silica erosion/days	*t*-ex Pt release/days
Ca(−)1-d30	222	57
Ca(−)2-d30	270	54
Ca(+)1-d30	36	6
Ca(+)2-d30	35	3

Table 2 Percentages of complex **3** released in the initial burst or left embedded in the xerogels (loading capacity). The determinations were performed 1 (d1) and 30 (d30) days after preparation

Formulation	% Burst	Pt(II) complex released in the burst/mg g^{-1} xerogel	Pt(II) complex left embedded in the matrix after the burst/mg g^{-1} xerogel
Ca(−)1-d1	31	0.59	1.31
Ca(−)1-d30	18	0.35	1.55
Ca(−)2-d1	41	1.22	1.76
Ca(−)2-d30	21	0.63	2.35
Ca(+)1-d1	36	0.45	0.79
Ca(+)1-d30	30	0.38	0.86
Ca(+)2-d1	60	0.94	0.63
Ca(+)2-d30	60.5	0.95	0.62

The nature of the released species was investigated by UV-Vis spectroscopy. A highly significant difference was observed between the Ca(−) and Ca(+) formulations. The Pt species released from the Ca(−) gel always displayed a UV spectrum similar to that of the initially entrapped dinuclear complex **3** (data not shown), demonstrating that the species released in the initial burst and in the subsequent slow process is the same as the one embedded. On the contrary, the Pt species released from the Ca(+) xerogel had UV spectra depending upon which phase of the release process is analyzed. In particular, the compound released in the initial burst had UV spectrum similar to that of the initially entrapped complex **3**, whereas the compound released in the second phase had UV spectrum with a maximum of absorbance at 300 nm, which is similar to that of $[PtCl_2(en)]$ under similar conditions. These results clearly indicate that calcium acts as an anchoring moiety for the bisphosphonic ligand while the released Pt compound is either $[PtCl_2(en)]$ (formed by reaction with chloride ions present in the buffer) or related aqua species. The pharmacological relevance of this finding is related to the different therapeutic potency of the two Pt species involved ($[Pt_2(en)_2(AHBP\text{-}H)]^+$ and $[PtCl_2(en)]$) and is currently under investigation.

It is worth noting that the release experiments were carried out under sink conditions always allowing fresh buffer to be in contact with the platinum-loaded xerogel. These conditions are likely to mimic the *in vivo* situation where constant renovation of body fluids occurs around the implant. It is important to point out that the concentration of Pt in the medium never reached the therapeutic value (even assuming that the released compound had similar potency as cisplatin). This means that the therapeutic activity of the matrices here investigated can be exerted only locally at their surface, while the surrounding tissue will not be affected.

We also want to point out that the trend observed in the present study (calcium acting as an anchoring moiety for a bisphosphonic ligand coordinated to a platinum atom) is very similar to what we have found in a previous study conducted on a Pt-complex with medronate ($[Pt_2(en)_2(MDP)]$; en = ethylenediamine, MDP = methylenebis(phosphonate)) embedded into two biomimetic synthetic hydroxyapatite nanocrystalline materials.[19] We found that the release of platinum from the hydroxyapatite composite materials occurred through complete breakage of the platinum–medronate bonds, the process depending upon the surface stoichiometry (Ca : P ratio).

Conclusions

In this work we have synthesized and fully characterized by multinuclear NMR spectroscopy a geminal aminobisphosphonic acid (**2**) and the corresponding platinum complex (**3**). The complex was found to have a dinuclear structure with the bisphosphonate bridging two platinum moieties in a W conformation. ^1H and ^{31}P NMR experiments at different pH values have allowed the determination of the acidity constants for the free aminobisphosphonic acid and an estimation of the stability of the Pt-complex. Complex **3** is stable at pH values not exceeding 8.0, which is compatible with carrying on pharmacological investigations.

The Pt(II) bisphosphonate complex was thus embedded into two different silica-based polymers to obtain biocompatible hybrid materials to be used for the local treatment of bone tumors. Our investigation is aimed at controlling the release properties of the hybrid material by changing the inorganic network composition, either pure silica or Ca^{++} added silica. The presence of calcium in the matrix was found to reduce its loading capacity but to improve its stability upon storage, a property that is fundamental for practical applications. In addition, the presence of calcium affected the nature of the Pt complex released in the slow diffusion-controlled process following the initial burst. When present, Ca^{++} was able to retain the bisphosphonic ligand so that only the Pt(en) residue was released from the xerogel. In contrast, in the absence of calcium, the Pt complex was released in its original dinuclear form with bridging bisphosphonate. Hopefully, the Pt complex concentration will be sufficient to exert therapeutic activity only at the site of the implant while it will be too low to exert undesired toxic effects on the neighbouring tissues and at a systemic level. We are currently performing cytotoxicity tests on both the dinuclear platinum complex and the platinum-embedded silica xerogels to investigate how the different nature of the released platinum species affects the biological activity.

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