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Osteogenesis promoted by calcium phosphate *N*,*N*-dicarboxymethyl chitosan

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Abstract

The effects of *N*,*N*-dicarboxymethyl chitosan (DCMC) on the precipitation of insoluble calcium salts, namely phosphate, sulfate, oxalate, carbonate, bicarbonate and fluoride, and magnesium salts, namely phosphate and carbonate, were studied. Results indicated that the chelating ability of DCMC interfered effectively with the well-known physico-chemical behaviour of magnesium and calcium salts. Dicarboxymethyl chitosan formed self-sustaining gels upon mixing with calcium acetate, as a consequence of calcium chelation. DCMC mixed with calcium acetate and with disodium hydrogen phosphate in appropriate ratios (molar ratio Ca/DCMC close to 2.4) yielded a clear solution, from which, after dialysis and freeze-drying, an amorphous material was isolated containing an inorganic component about one half its weight. This compound was used for the treatment of bone lesions in experimental surgery and in dentistry. Bone tissue regeneration was promoted in sheep, leading to complete healing of otherwise non-healing surgical defects. Radiographic evidence of bone regeneration was observed in human patients undergoing apicectomies and avulsions. The DCMC–CaP chelate favoured osteogenesis while promoting bone mineralization.

Keywords: Osteogenesis; *N*,*N*-dicarboxymethyl chitosan; Calcium chelation; Carboxymethylation; Bone regeneration; Hydroxyapatite

1. Introduction

Chitin is often found in association with $CaCO_3$ in insects and arthropods. On the other hand, the mineralized tissues of vertebrates contain crystalline hydroxyapatite. Vesicles containing calcium ions and phosphate ions in equilibrium with complexing compounds give rise to plates adjacent to collagen fibrils, typically 35 × 8 × 1.5 nm for tendon (Erts et al., 1994) or 50 × 25 × 3 nm for bone (Zhang & Gonsalves, 1995). Biological hydroxyapatites exhibit low crystallinity and lack stoichiometry due to the presence of adsorbed ions or included ions (Bigi et al., 1992).

The inorganic component of these tissues is formed in the presence of various substances exerting inhibiting actions and structural alterations; for instance, the reaction

$$2CaHPO_4 + 2Ca_4(PO_4)_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (38°C)$$

is exposed to the influences of macromolecular species (e.g. albumin), ionic species (e.g. bicarbonate ion) and other species which produce important alterations (e.g. the hydroxyapatite carbonation) (Martin & Brown, 1994).

The treatment of bone lesions with the aid of granular hydroxyapatite is relatively simple, because hydroxyapatite is the ultimate product of the mineralisation process *in vivo*. The hydroxyapatite granules of artificial hydroxyapatite are not valid surrogates of the nanocrystals generated *in vivo*; instead, they slow down the cellular proliferation. Porous hydroxyapatites and coated hydroxyapatites were developed to obviate these drawbacks (Eggli et al., 1987).

Various formulations for the preparation of hydroxyapatite-based bone cements have been described by Driessens

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et al. (1994), Akai (1994) and Ito et al. (1994). Cements containing chitosans have been proposed by Sumita (1988) who mixed tricalcium phosphate and tetracalcium phosphate with chitosan acetate (Lacout et al., 1996). Takechi et al. (1996) suggested that the cement stability is due to the impermeability promoted by chitosan and underline the advantage of the modest exothermic effect (about 33°C, compared to about 115°C for poly(methyl methacrylate) (Maruyama & Ito, 1996). Ishii (1992) mixes $Ca_3(PO_4)$ with chitosan.

Wherever a cement is not strictly necessary, materials conceived in a totally different way might be more desirable. A resorbable hydroxyapatite is a compound that osteoblasts can restructure: for example, the inorganic compound could include intercalated biopolymers in the crystal lattice (Messersmith & Stupp, 1992). Lohman (1993) discusses the preparation of NaCl, $CaSO_4$ and $CaCO_3$ in the presence of various biopolymers but does not characterize the resulting materials. At the present time these materials are not available.

While chitosan and many of its derivatives have chelating ability towards transition metal ions but not for alkali metal ions (Muzzarelli & Tubertini, 1969), chitosans carrying carboxyl groups might chelate calcium (Muzzarelli and Zattoni, 1986; Muzzarelli, 1988; Chiessi et al., 1992). Rinaudo et al. (1992) and Muzzarelli et al. (1985 and 1994) have produced and fully described highly carboxymethylated chitosans about which, however, nothing is known in terms of their effect on the crystallization of sparingly soluble salts. Gruber (1996) prepared *N*[(3′-hydroxy-2′,3′-dicarboxy)ethyl]chitosan for cosmetic purposes. On the other hand, Inoue et al. (1995) and (1996) synthesized a class of chitosans carrying chelating EDTA-type functional groups and used them for the separation of lanthanons. Related compounds are (2-carboxyethyl)chitosan (Shigemasa et al., 1995) and partially dicarboxylated chitosan obtained by periodate and chlorite oxidation by Matsumura et al. (1997).

Ina & Nakamura (1992a,b) proposed carboxymethylated chitosans for the preparation of detergents, and other authors considered analytical aspects (Menon et al., 1995; Rinaudo et al., 1992; Guibal et al., 1995), but so far no bioinorganic material of this class has been reported.

The purpose of the present work was to verify the hypothesis that homogeneous association of highly carboxymethylated chitosans and calcium phosphate might promote not only the formation of bone tissue but also its mineralization: in fact, the osteogenetic capacities of chitosan have been reported recently by Muzzarelli et al. (1993) and Muzzarelli (1997) and by Klokkevold et al. (1996).

2. Experimental

2.1. Chitosans

Crustacean chitosan manufactured by Aber Technologies (Plouvien, France) was supplied by Merck Clevenot (Nogent-sur-Marne, France). *N*,*N*-Dicarboxymethyl chitosan (DCMC) was prepared according to Le Dung et al. (1994) as described briefly below. The concentrations indicated are crucial parameters and should be adopted if a fully disubstituted chitosan is to be prepared. DCMC was prepared under the following conditions: the chitosan powder (30 g) was suspended in demineralized water (3 l); glacial acetic acid (27 g) was added to dissolve the chitosan and after stirring for 20 min, glyoxylic acid was added (178 ml 50% v/v corresponding to 119 g of glyoxylic acid). The molar ratio of amine/glyoxylic acid was 1:9. The final pH was 2–3. Sodium borohydride (90 g) in water (2.5 l) was delivered as a 3.6% solution to the reaction vessel using a peristaltic pump (1.2 ml min^{-1}). DCMC showed scarce solubility at pH 4.5 but spontaneous dissolution took place with further delivery of borohydride. The final pH was 5.5; the clear solution was dialysed against demineralized water for 36 h, in dialysis tubing with a cut-off value of 2500 Da.

In cases where the dialysis lasted longer than 36 h, some precipitate was observed, which dissolved upon addition of 0.1 M ammonia (final pH 6.0). Finally, the solution was freeze-dried. The products for *in vivo* studies were sterilized by γ-ray irradiation at 25 kGy and the aspect was that of a soft spongy and hydrophilic material.

2.2. Insoluble salts

Analytical grade reagents were used to precipitate the insoluble salts according to the standard procedures of qualitative analytical chemistry.

2.3. Animals

The study was performed in sheep in view of the histological, physico-mechanical and physiopathological similarity to human femurs. The animals were treated according to the recommendations of the European Economic Community. The day before surgery, 100 mg kg^{-1} of Cefazolin (Cefamezin) were administered. The animals underwent surgery after starving for 12 h. Following a premedication with ketamine (10 mg kg^{-1}) and xylazine (0.2 mg kg^{-1}), general anaesthesia was induced with thipethane-natrium (8 mg kg^{-1}, 2.5% solution) and maintained with a gas mixture of 0.5–1.0% halothane and $N_2O + O_2$ (1:l) in automatic ventilation. A bone defect was created in the femural epiphyses by means of a 6 mm drill. During surgery the drill holes were carefully rinsed with 0.9% NaCl solution and cleaned out in order to remove abraded particles, reduce drilling temperature and avoid bone necrosis. In the right leg the hole was completely filled with IMIC–chitosan or TCP–chitosan, while in the other one it was left open to serve as control. The wounds were sutured atraumatically in two layers and disinfected. Animals were submitted to antibiotic therapy (25 mg kg^{-1}

cefazolin pro die) for 7 days, and were allowed to bear weights as tolerated. Neither intra- and post-operative nor general and local septic complications were observed. Animals were killed after 40 and 60 days under general anaesthesia as above. Femurs were removed and sawn to produce fragments for histological examination. Specimens were fixed in 10% buffered formalin, dehydrated in an ascending series of alcohol and embedded in methacrylate resin. After polymerization, blocks were sectioned along a plane perpendicular to the bone surface by using a diamond saw and yielding undecalcified ground sections of thickness 100 μm. Stained sections were examined using light microscopy and polarized-light microscopy.

2.4. Instrumental analytical techniques

X-ray diffraction spectra were obtained by using a vertical powder diffractometer; the source was a rotating anode generator Rigaku Denki RU-300 and Ni-filtered Cu Kα radiation ($\lambda = 0.154$ nm) was used.

Metal ions were determined by atomic absorption spectrometry with a Perkin-Elmer 2380 spectrometer.

The infrared spectra were obtained with a Nicolet 20-SX FTIR spectrometer (DTGS detector) equipped with a Spectra Tech. Multiple Internal Reflectance (DRIFT) accessory for measurements in the solid state. For spectral determinations, the micro cup of the accessory was filled with a mixture of a small amount of sample ground with anhydrous KBr. Resolution was 4 cm^{-1}; 250 scans; smoothing from 13 to 25. Data handling was done with a Galactic software package: smoothing from 13 to 25; the curvefit, after polynomial baseline correction had been made assuming a Lorentzian character. Attribution of the bands was based on literature data (Schrader, 1995; Bruni et al., 1997).

A Philips SEM 505 scanning electron microscope was also used. The samples were prepared after cutting them to expose the inner part, fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h, dehydrated and submitted to critical point drying and gold coated; controls, simply gold coated, showed no difference thus excluding artifact formation.

3. Results and discussion

The presence of DCMC in the aqueous media where reactions of magnesium and calcium ions take place strongly affects the yield and crystallinity of the insoluble salts, to the point that conditions can be defined where no precipitation occurs at all.

Table 1 summarizes the macroscopic observations for a number of anions in water at the specified pH values when reacted with calcium ions, in the presence of DCMC. For selected weight ratios, specified below, it is possible to observe that, in the presence of DCMC, calcium phosphate does not precipitate while calcium sulfate and calcium bicarbonate do: this behaviour diverges from the regular behaviour of calcium salts in the absence of the biopolymer.

Table 1
Observations on the reactions between calcium acetate and sodium salts, in the absence and presence of DCMC, at concentrations specified in the text

Sodium salt	Final pH	General aspect	
		DCMC present	DCMC absent
Phosphate	6.1–6.2	Clear, homogeneous	Precipitate
Sulfate	5.9–6.7	Turbid	Clear, homogeneous
Oxalate	5.8–6.6	Turbid	Precipitate
Carbonate	8.3–9.0	Turbid	Precipitate
Bicarbonate	7.2–9.0	Turbid	Clear
Fluoride	6.2–6.9	Precipitate with a clear supernatant	Turbid

When DCMC is reacted with calcium ions, the reaction results in products of various aspect, depending on the weight ratio Ca/DCMC, as shown in Table 2. In practice, precipitation occurs for large ratios, and no precipitate is observed for small ratios; but for ratio 0.011 a rigid gel forms, that assumes the shape of the container and can be sliced with a knife. The gel of DCMC–Ca dissolves upon introduction into a disodium orthophosphate solution, yielding a limpid solution. No pH (5.5) variation is observed.

In order to identify the most suitable conditions for the formation of the water-soluble DCMC–CaP complex, the investigation presented in Table 3 was carried out. It can be seen that, for equimolar quantities of disodium hydrogen orthophosphate and calcium acetate, a weight ratio Ca/DCMC of 0.4 provided a homogeneous system. These conditions correspond in practice to 0.254 g DCMC + 0.26 g phosphate ion + 0.11 g Ca(II), the latter corresponding to 0.48 g of Na_2HPO_4 and 0.52 g $Ca(CH_3COO)_2$. From this system a water-soluble DCMC–CaP complex was isolated: this product was soluble in a wide pH range. Nevertheless, NH_4OH at pH 8 permitted an easy isolation of the polymer from the mother liquor. When the preparation of the water-soluble DCMC–CaP complex was made starting from the

Table 2
Observations on the precipitate formation from DCMC (5 g of a 0.56% solution, constant quantity) and calcium acetate (1.0%), at 20°C. Constant pH for all assays 5.5

Calcium (mg)	Weight ratio Ca/DCMC	Observations
0.06	0.0021	No precipitate
0.08	0.0029	No precipitate
0.10	0.0036	Turbidity
0.21	0.0075	Precipitate
0.31	0.0110	Gel/precipitate[a]
1.04	0.0371	Gel/precipitate
2.08	0.0743	Precipitate
3.08	0.1100	Precipitate
5.12	0.1829	Precipitate

[a]For this weight ratio a white, rigid and self-sustaining gel formed and took the shape of the container.

Table 3
Observations on the precipitate formation subsequent to the addition of a constant quantity of calcium acetate (130 μmol) to a mixture of DCMC (variable quantity) with disodium hydrogen phosphate (130 μmol) at 20°C. Constant pH for all assays 6.1

DCMC		Weight ratio Ca/DCMC	Observations
grams of solution 0.56%	mgrams		
0.1	0.56	9	Precipitate[a]
0.2	1.1	4.7	Precipitate[a]
0.3	1.7	3	Precipitate[a]
0.4	2.2	2.4	Precipitate[a]
0.5	2.8	1.9	Precipitate[a]
0.6	3.3	1.6	Precipitate
1.2	6.7	0.7	Precipitate
2.1	11.8	0.4	Clear
3.1	17.4	0.3	Precipitate
3.7	20.7	0.25	Precipitate
4.4	24.6	0.21	Precipitate
5.0	28	0.18	Precipitate

[a]These precipitates were dried at 40°C and submitted to X-ray diffraction analysis (see Figure 1B).

Table 4
Observations on the precipitate formation subsequent to the addition of a constant quantity of calcium acetate (130 μmmol) to a mixture of ammonia-treated DCMC (variable quantity) with disodium hydrogen phosphate (130 μmol), at 20°C. Constant pH for all assays 6.0

DCMC		Weight ratio Ca/DCMC	Observations
grams of solution 0.38%	mgrams		
1	3.8	1.3	Precipitate
2	7.6	0.7	Precipitate
3.1	11.8	0.4	Precipitate
3.4	13	0.4	Precipitate
4	15	0.35	Precipitate
4.4	16.7	0.31	Precipitate
5	19	0.27	Turbid
5.4	20.5	0.25	Turbid
6	22.8	0.22	Turbid
6.5	24.7	0.21	Clear
7	26.6	0.20	Clear
7.5	28.5	0.18	Clear
8	30.4	0.17	Clear

ammonia-treated solution (see Section 2) the Ca/DCMC weight ratio was 0.21, somewhat lower than 0.4, as indicated in Table 4.

3.1. X-ray diffraction

At the X-ray analysis, the water-soluble DCMC–CaP complex was amorphous. The samples prepared according to the conditions shown in Table 3 and dried at 40°C showed scarce crystallinity (Fig. 1). Most of the diffraction bands were depressed or absent. The typical peaks for brushite at 2θ 11.68, 20.96 and 29.28, intense in the control precipitated under identical conditions, were strongly depressed in the samples. Of the remaining peaks, only that at 2θ 4.64 depended on the DCMC concentration. The spectra showed that even minor amounts of DCMC present in the reaction medium led to nearly amorphous associations of DCMC and $Na_2HPO_4 \cdot 2H_2O$.

3.2. Electron microscopy

DCMC is a filmogenic substance, that forms transparent and mechanically resistant films. Even in the spongy freeze-dried DCMC, the tendency to form films and filaments is quite evident when the material is examined at the electron microscope (Fig. 2).

On the contrary, the DCMC–CaP complex does not keep the filmogenic properties of the parent DCMC, because the material in the dry state is fragile and can be crushed easily. An electron micrograph is in Fig. 3.

3.3. Elemental analysis

The chemical derivatization introduced carbon, oxygen and sodium into the polysaccharide with a resulting N/C ratio for DCMC (Na form) of 0.115, well below the N/C ratio for plain chitosan (0.182). The sum N + C + H for DCMC was lower than 40% while for plain chitosan it was close to 60%.

In the complex DCMC–CaP, the nitrogen percentage dropped to less than one half (1.66 for DCMC–CaP instead of 3.53 for DCMC) and therefore the ratio organic/inorganic in the complex was about 1:1 by weight, as was also confirmed by the analysis of the ashes at 600°C. The elemental analyses were as follows: DCMC, C 30.59, N 3.53, H 4.70, Na 10.0, N/C 0.115; DCMC–CaP, C 21.68, N 1.66, H 3.51, Ca 15, P 12, N/C 0.076; parent chitosan[0.20], C 44.71, N 8.26, N/C 0.182.

3.4. IR spectrometry

In the 3600–3000 cm^{-1} region, both DCMC and DCMC–CaP exhibit a highly convoluted infrared band due to various OH stretching contributions. The infrared spectra of the two compounds, in the 1800–900 cm^{-1} region, are shown in Fig. 4. The asymmetric and symmetric stretching modes of the carboxylate group are represented by two composite bands with maxima at 1596 and 1406 cm^{-1} in DCMC sodium salt (1591 and 1413 cm^{-1} in DCMC–CaP), respectively. In DCMC–CaP, the phosphate vibrations are overlapped with bridge oxygen and C–O stretching modes and cannot be easily isolated (Fig. 4). A comparison of curvefit results of the latter band with those from DCMC allowed to us to characterize the phosphate contributions (Fig. 5). The percentage of band area of phosphate with respect to sugar backbone (taking the C–H stretching at 2878 cm^{-1} as internal standard) resulted in around 50%, in agreement with the elemental analysis data.

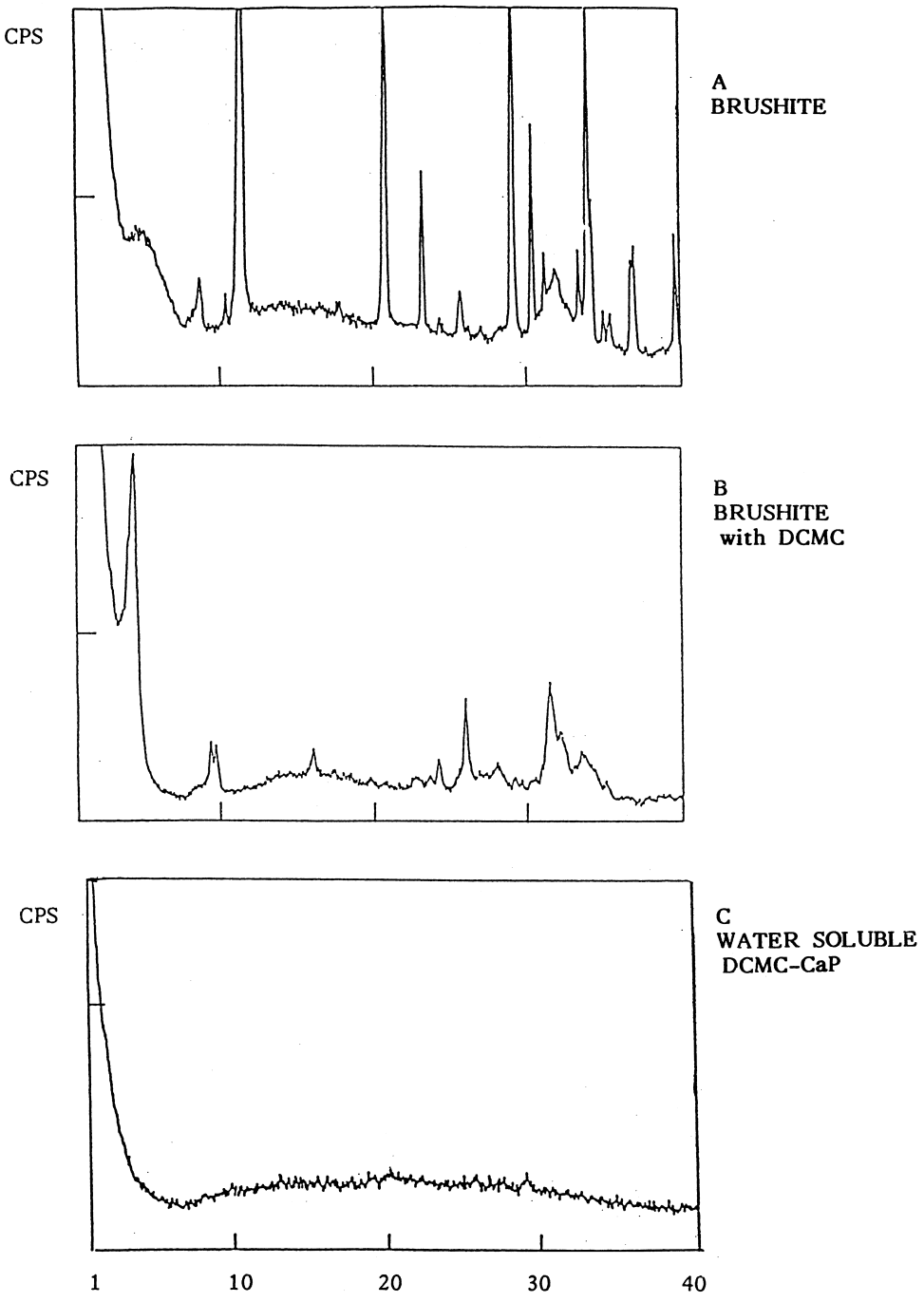

Fig. 1. X-ray diffraction spectra recorded on samples dried at 40°C. (A) Control sample of calcium phosphate precipitated from $Ca(CH_3COO)_2$ and Na_2HPO_4 solutions, showing diffraction peaks typical of brushite. (B) Sample precipitated in the presence of DCMC (line 5 in Table 3) exhibiting greatly depressed diffraction peaks whilst the one at 2θ 4.62 is enhanced. (C) Water-soluble, homogeneous sample (prepared according to line 8 in Table 3) exhibiting complete absence of crystallinity.

3.5. Recommended preparative conditions

The DCMC–CaP complex was prepared as follows. DCMC (0.48%, 220 g) was mixed with $Na_2HPO_4 \cdot 2H_2O$ (1.0%, 67 ml) and calcium acetate (1.0%, 72 ml). The quantities were 0.6 g of $Na_2HPO_4 \cdot 2H_2O$ and 0.7 g of calcium acetate per gram of DCMC. After dialysis and freeze-drying, the material yielded a gel or a solution when mixed with water or saline, depending on the product to water ratio.

3.6. Other sparingly soluble salts

In addition to phosphate, we also tested sulfate, oxalate, carbonate, bicarbonate and fluoride for their capacity to form calcium salts in the presence of DCMC. Magnesium

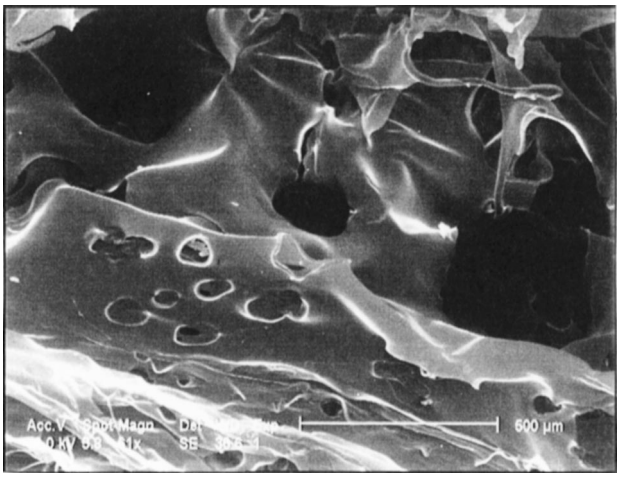

Fig. 2. Electron micrograph for a DCMC freeze-dried sample, providing evidence of the filmogenic capacity and large surface availability.

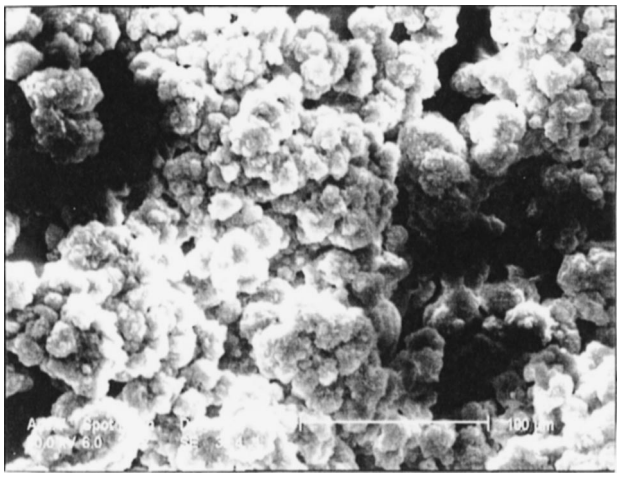

Fig. 3. Electron micrograph for a DCMC–CaP freeze-dried sample providing evidence of the disordered formation of calcium phosphate in the presence of DCMC.

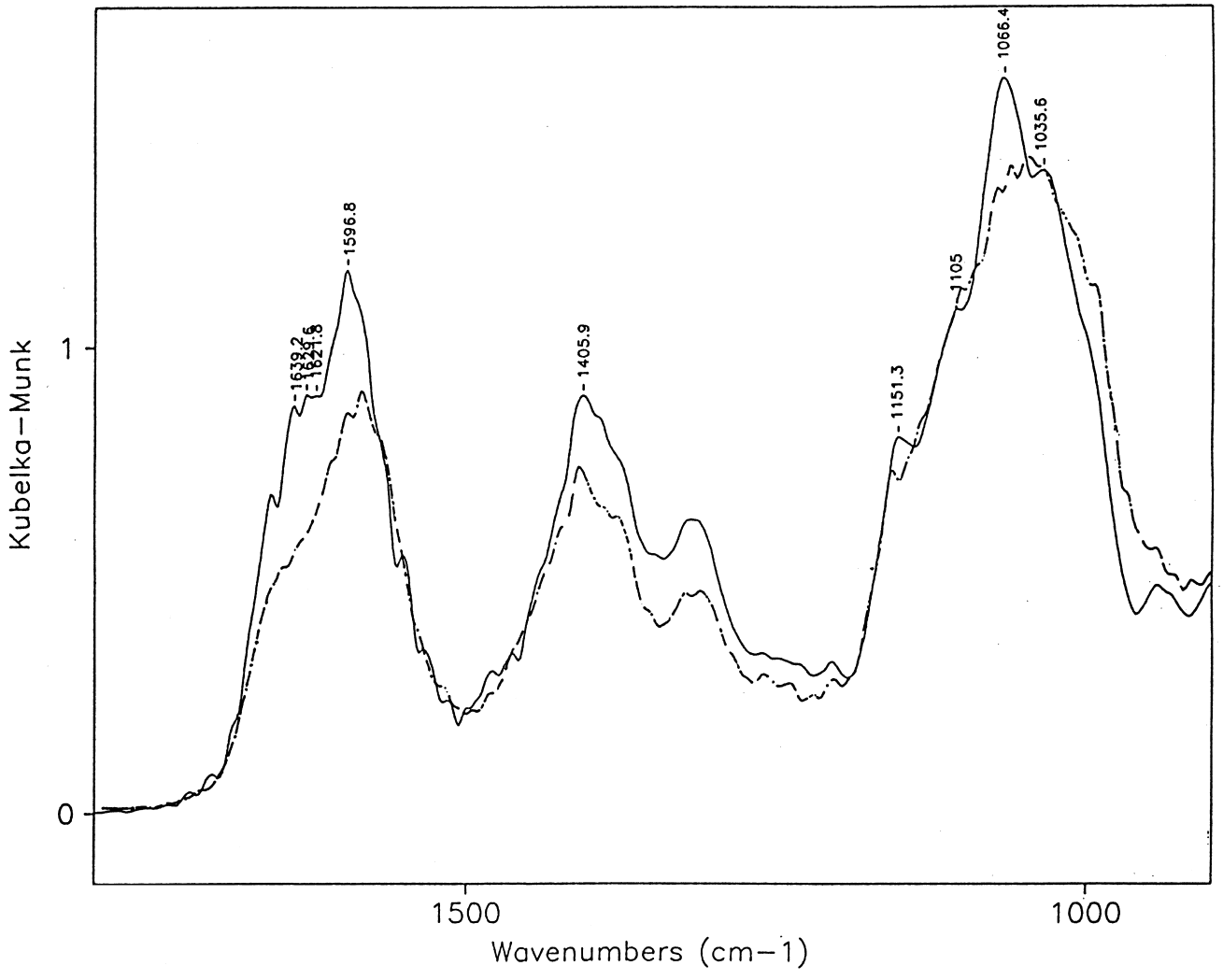

Fig. 4. Infrared spectra for DCMC (————————) and for DCMC–CaP (- - -).

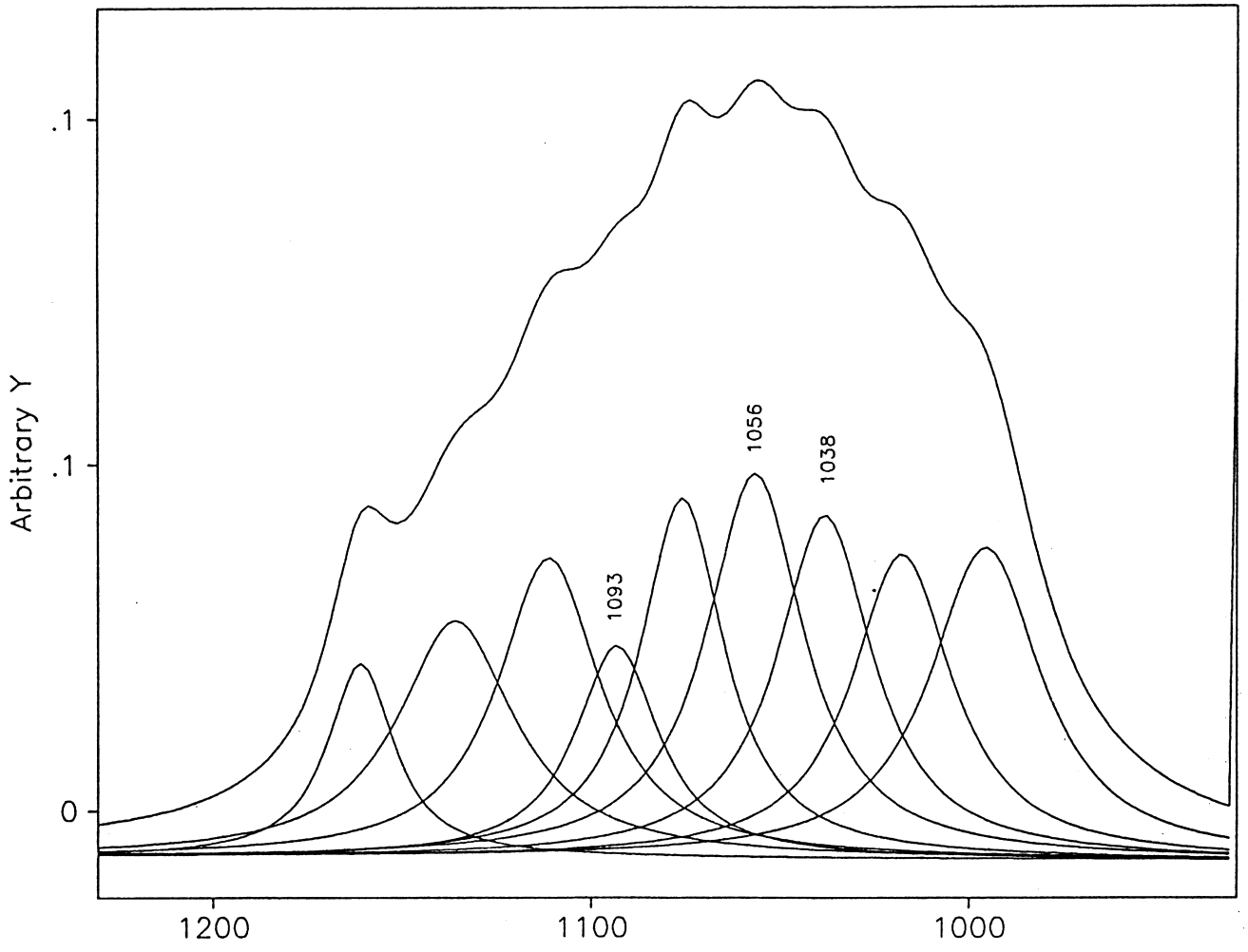

Fig. 5. Curvefit of the band at 1230–930 cm^{-1} in DCMC–CaP. The main phosphate contributions are found at 1093, 1056 and 1038 cm^{-1}.

phosphate and carbonate were studied as well. Generally speaking, the DCMC affected their precipitation macroscopically, and influenced the crystallinity of the products even more.

Calcium sulfate (degree of solubility 1.1 g l^{-1}; solubility product 6.1×10^{-5}) did not precipitate in the controls upon mixing ammonium sulfate and calcium acetate at pH 5.9–6.7, but a fluffy precipitate was observed for Ca/DCMC weight ratios in the range 0.17–1.15, whose bulkiness depended on the DCMC concentration.

Calcium oxalate (degree of solubility 5.7×10^{-3} g l^{-1}; solubility product 2×10^{-9}) precipitated in the controls in the pH range 5.8–6.6; in the presence of DCMC the supernatants were turbid for a Ca/DCMC weight ratio in the range 0.17–0.23 while for higher ratios the supernatants were clear.

Calcium carbonate (degree of solubility 1.3×10^{-2} g l^{-1}; solubility product 1.7×10^{-8}) precipitated in the controls in the pH range 8.3–9.0; in the presence of DCMC, however, no real precipitate was observed in spite of the fact that all solutions turned turbid in the Ca/DCMC weight ratio 0.17–6.8. Therefore, DCMC prevented the precipitation of $CaCO_3$.

Calcium bicarbonate, a soluble salt, in the presence of DCMC yielded increasing turbidity with decreasing Ca/DCMC weight ratio (0.17–6.8) in the pH range 7.2–8.0.

Calcium fluoride (degree of solubility 1.6×10^{-2} g l^{-1}; solubility product 3.2×10^{-11}) precipitated in the controls in the pH range 6.2–6.9 and did not sediment promptly; in the presence of DCMC the supernatants became clear after 1 day for Ca/DCMC weight ratios in the range 0.17–0.34. These materials, once desiccated at 55°C, became hard and yellowish, especially those with higher DCMC content. In the X-ray diffraction spectra for these compounds (DCMC–CaF) the single (111) peak at 2θ 28.34 typical for CaF_2 showed intensity inversely proportional to the DCMC concentration.

The addition of magnesium nitrate to mixtures of DCMC with $Na_2PO_4 \cdot 2H_2O$ or with Na_2CO_3 (Table 5 and Table 6, respectively) promoted precipitation of insoluble salts for weight ratios Mg/DCMC higher than 0.9 and 2.2, respectively. In the presence of larger Mg concentrations

Table 5
Observations on the precipitate formation subsequent to the addition of a constant quantity of magnesium nitrate (140 μmol) to a mixture of DCMC (variable quantity) with disodium hydrogen phosphate (140 μmol), at 20°C

DCMC		Weight ratio Mg/DCMC	Observations
grams of solution 0.156%	mgrams		
0.1	0.156	21.8	Precipitate
0.5	0.78	4.4	Precipitate
1.0	1.56	2.2	Precipitate
2.0	3.12	0.9	Clear

no precipitation took place, not even after several days at 20°C.

3.7. Bone tissue regeneration in animals

In sheep medicated with DCMC–CaP, macroscopic analysis evidenced an irregular rimmed area smaller than the surgical defect, filled with a tissue without the histoarchitectural characteristic of bone tissue. In control femurs the hole had not changed much in shape and size since surgery and the area lacked bone tissue. Microscopic analysis of treated legs clearly showed the difference between the reparative–reconstitutive bone tissue with or without chitosan. First of all, the number of cells were higher in the treated legs than in the control ones, and their large size and star-shape were suggestive of activation. However, the most important factor was the presence of a wide osteogenic reaction (Fig. 6) moving from the rim of the surgical lesion toward the centre. Vascular endothelial cells synthesize and secrete an array of soluble mediators either constitutively or in response to induction stimuli such as injury or inflammation. Among these, it is important to mention growth factors and cytokines: fibroblast growth factor (FGF), interleukin-1 (IL-1), interleukin-6 (IL-6), colony simulating factors (CSFs) of the G, GM and M subtypes, arachidonic acid metabolites like prostacyclin, and small peptides like endothelin-1. These regulatory compounds have been seen, in other studies, to control the recruitment, proliferation, differentiation, functioning, and/or survival of various cells including bone-forming osteoblasts and bone-degrading osteoclasts. Lastly, endothelial cell release of other short-lived messenger molecules, namely reactive oxygen species and nitric oxide, may help to control osteoclast activity. In fact, activated oxygen species seem to enhance resorption, whereas NO has been shown to interfere with osteoclast bone resorption and, furthermore, inhibitors of NO synthethase potentiate osteoclast bone-resorption.

Table 6
Observations on the precipitate formation subsequent to the addition of a constant quantity of magnesium nitrate (140 μmol) to a mixture of DCMC (variable quantity) with sodium carbonate (140 μmol), at 20°C.

DCMC		Weight ratioMg/DCMC	Observations
grams of solution 0.156%	mgrams		
0.1	0.156	21.8	Precipitate
0.5	0.78	4.4	Precipitate
1.0	1.56	2.2	Clear

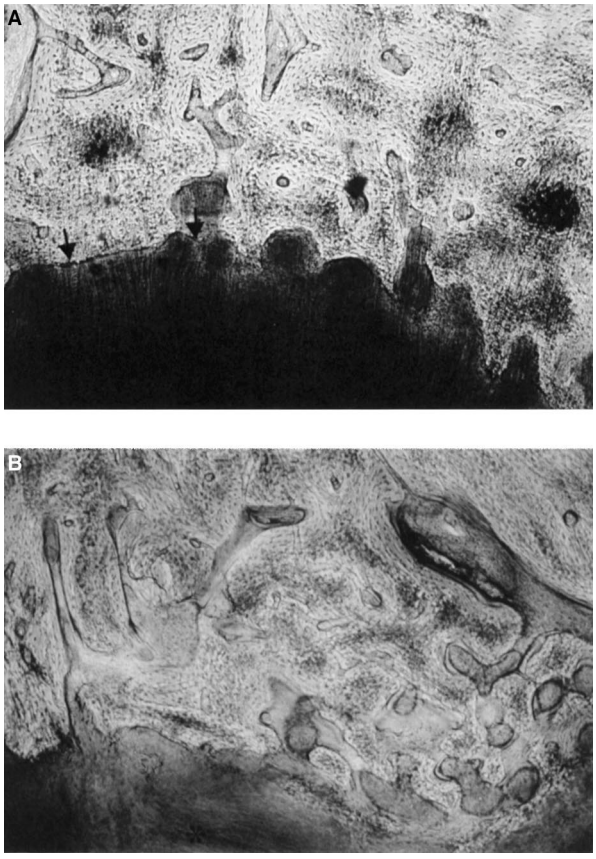

Fig. 6. Histological evidence of osteogenesis promoted by DCMC–CaP in sheep. (A) Control femoral trochanter 60 days after surgery: the surgical hole (lower dark part) displays a flattened rim (→) (× 500). (B) Chitosan treated femoral trochanter 60 days after surgery: wide bone remodelling at the periphery of the hole (lower part) (× 500).

The presently available data indicate the important role of DCMC in stimulating bone tissue reconstitution and suggest that its action can be potentiated by chelation of calcium phosphate.

3.8. Use of DCMC in dentistry

The avulsion of a dental unit and a cyst in a 15-year-old patient was performed: the space left was filled with freeze-dried DCMC–CaP. Radiographic observations, in Fig. 7, showed precocious formation of osseous trabeculae 15 days a.s. These data were supported by similar results in a number of apicectomies and avulsions.

4. Conclusions

DCMC exhibited chelating capacity for magnesium and

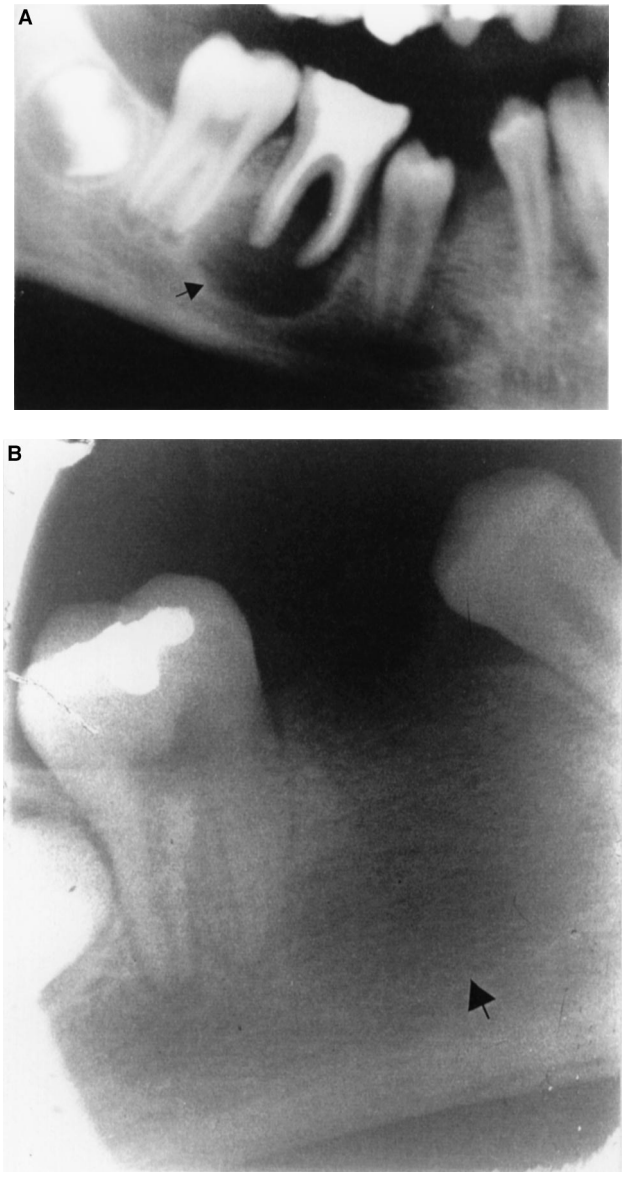

Fig. 7. Radiological evidence of osteogenesis promoted by DCMC–CaP in a 15-year-old patient. (A) Presence of a large cyst (→), dental unit 46, before surgery. (B) Precocious formation of bone tissue (→) 14 days after avulsion and cyst removal.

calcium and produced a gel when mixed with calcium acetate in appropriate proportions. Soluble chelates were formed upon mixing DCMC-bearing calcium solutions with phosphate. Sulfate, oxalate, carbonate, bicarbonate and fluoride solutions in the presence of DCMC also exhibited unpredicatable behaviour as far as calcium salts are concerned. The precipitation of magnesium phosphate and carbonate was inhibited by modest concentrations of DCMC.

The DCMC–Ca and DCMC–Mg chelates are novel compositions described here for the first time, which can be easily prepared and isolated for medical applications.

DCMC–CaP is a gel-forming material particularly suitable for conveying amorphous calcium phosphate to the bone defect site: the inorganic component is available promptly for transformation into physiologically valid hydroxyapatite.

DCMC–CaP possesses the osteoinductive properties of chitosans and the mineralisation capacities of an amorphous calcium phosphate associated with a biopolymer of high biochemical significance.

It has been verified experimentally that bone defects otherwise impossible to heal were healed promptly and re-mineralized in the sheep. DCMC–CaP was also advantageously used in apicectomies, avulsion sites dressing, and other bone restoration operations on patients.

Both DCMC and DCMC–CaP also lend themselves to coating of porous hydroxyapatite for better osteointegration when the use of artificial hydroxyapatite is preferred. DCMC was also associated with BMP (Muzzarelli et al., 1997). DCMC interferes in general with the precipitation of insoluble calcium salts; therefore, in consideration of its chelating ability with calcium, a number of other applications can be foreseen.

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