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In vivo and in vitro biodegradation of oxychitin–chitosan and oxypullulan–chitosan complexes

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Abstract

Oxychitin–chitosan complexes prepared from crustacean chitin and oxypullulan–chitosan complexes prepared from three different preparations of *Aureobasidium pullulans* pullulan were contacted with solutions of egg white lysozyme, *Carica papaya* papain, wheat germ lipase, *Clostridium histolyticum* collagenase, porcine pancreas α-amylase, barley malt α-amylase, and sweet potato α-amylase at nearly neutral pH values and 25 and 37°C, for at least three days. The reducing capacity of the solutions in contact with the complex, due to oligomer release, was measured with the aid of ferricyanide and expressed as net absorbance vs time. The oxychitin–chitosan complex was degraded by lysozyme, lipase and papain. The other enzymes were ineffective. Histological evidence indicated that the oxychitin–chitosan complex tested as a bone prosthesis coating in an animal model, was biochemically active and biodegradable, therefore capable to promote osteoconduction and greater bone formation at the bone-prosthesis interface, with no adverse effect on mineralization. Pullulans of different origins were susceptible to enzymatic hydrolysis, particularly so with animal amylase, nevertheless the chitosan complexes obtained from the corresponding oxypullulans were not degraded over the three-day observation period.

Keywords: Chitin; Chitosan; Pullulan; Oxypullulan–chitosan complex; Oxychitin–chitosan complex; Hydrolases; Enzymatic degradation

1. Introduction

According to recent literature, modified chitins undergo hydrolysis in the presence of a number of human, plant and animal enzymes (Muzzarelli, Stanic & Ramos, 1999b; Jollès & Muzzarelli, 1999; Muzzarelli, Xia, Tomasetti, & Ilari, 1995; Muzzarelli, 1992; Kurita, Youshino, Nishimura & Ishii, 1993; Kurita, Kaji, Mori & Nishiyama, 2000), but little is known about the biodegradability of their complexes. 6-Oxychitin, a polyanionic form of chitin, reacts with polycationic chitosan to form stable, insoluble complexes (Muzzarelli, Muzzarelli, Cosani & Terbojevich, 1999a) that under convenient conditions are amenable to microcapsules or microspheres suitable for delayed drug delivery (Muzzarelli et al., 2000).

Other polyanion–chitosan complexes are well assessed since long: they include, heparin (Kubota & Kikuchi, 1998), alginate (Lee, Park & Ha, 1997), gellan (Amaike, Senoo & Yamamoto, 1998), mucin (Fiebrig, Varum, Harding, Davis & Stokke, 1997), carrageenan (Hugerth, Caram-Lelham & Sundelof, 1997), pectin (Hoagland and Parris, 1996; Meshali & Gabr, 1993), xanthan (Dumitriu, Magny, Montane, Vidal & Chornet, 1994), carboxymethyl cellulose (Yoshioka, Hirano, Shioya & Kako, 1990), carboxymethyl cyclodextrin (Furusaki, Ueno, Sakairo, Nishi & Tokura, 1996), scleroglucan (Guo, Elgsaeter, Christensen & Stokke, 1998), hyaluronic acid and chondroitin sulfate (Denuzière, Ferrier, Damour & Domard, 1998). This topic has been recently reviewed (Kubota & Kikuchi, 1998).

While plain oxychitin is rather reactive towards proteins and tends to precipitate some of them, ongoing research seems to indicate that its combinations with chitosan do not strongly react with enzymatic proteins. Therefore this material is becoming attractive for the mechanical stabilization of chitosan coatings on the surface of biomedical items, and for the modulation of the biodegradation of chitosan in other applications (Mattioli-Belmonte et al., 1999; Muzzarelli, Biagini, De Benedittis, Mengucci, Majni & Tosi, 2001).

An experimental model with ovariectomised osteoporotic rats would be useful to assess the resorption and the in vivo efficacy of the oxychitin–chitosan complex, based on the following rationale. Prosthetic implants cause free-radical production that jeopardises bone-prosthesis integration, particularly in patients with impaired reparative response; polysaccharides on the other hand are believed to enhance bioenergetic and metabolic bone homeostasis (Xue, Yu,

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Hirata, Terao & Lin, 1998). In particular, modified chitosans exert control over the cascade of free-radical production, thus favoring early bone integration and regeneration and avoiding primary biological failure (Ducheyene & Qui, 1999; Puleo & Nanci, 1999; Jones, Frondoza & Hungerford, 1999; Mancini, Moradi-Bidhendi, Brandi & MacIntyre, 1998).

Scope of the present article is to study the biodegradability of the oxychitin–chitosan complex in vivo, as well as in vitro, and to expand the knowledge on the enzymatic degradation of insoluble carbohydrates (Saddler & Penner, 1995).

Data on pullulans are of interest because of their proposed use as excipients. Their biosynthesis has been recently improved with the aid of selected strains of *Aureobasidium pullulans* and improved growth media (Gniewosz, Sobczak, Wojciechowska & Kuthan-Styczen, 1999; Kim, Kim, Lee, Lee and Kim, 2000; West & Strohfus, 1999). No information is available on their unspecific enzymatic hydrolysis.

The regiospecifically oxidized oxypullulan and the newly synthesized oxypullulan–chitosan complexes, here presented for the first time, are considered in order to provide a suitable comparative model for highlighting the peculiarities of the oxychitin–chitosan complex.

2. Materials and methods

2.1. Viscometry

The measurements were done at the shear rate value of 200 s^{-1} that did not generate excessive mechanical degradation of the polymers. The Haake Rotovisco RV-20 M5 was driven by a computer with Haake software. The thermostatic double-walled NV rotor was housed in an 11 ml cup filled with the solution under study.

2.2. Colorimetry

The method of Schales was used as described by Kurita et al. (1993). The freeze-dried polysaccharide (25 mg), the acetate buffer solution (0.1 M, optional, otherwise water, 50 ml) and the enzyme solution (25 ml containing 0.8 mg enzyme) were introduced in a 100 ml Erlenmeyer flask kept at 37°C; measurements were done on 3 ml aliquots withdrawn at fixed time intervals. Each aliquot was mixed with potassium ferricyanide solution (4 ml) prepared by dissolving 0.5 g of complex salt in 0.5 M Na_2CO_3 to 1 l.

The mixture was kept in a water bath at 100°C for 15 min, then cooled to 25°C within 5 min, and read at 420 nm with a Pharmacia LKB Novaspec II colorimeter.

The control solutions were the following:

1. 0.1 M acetate buffer,
2. enzyme solution (25 ml) with buffer solution (50 ml),
3. buffer solution (75 ml) with polysaccharide (25 mg).

The calculations were based on the following differences:

(A) 1–2 = absorbance due to the enzyme alone,
(B) test solution — 3 = absorbance due to released matter from the complex.

B − A = ΔAbs, net absorbance due to the enzymatic hydrolysis products of the polysaccharide, plotted vs time.

The largest error associated with these determinations was 0.4% on the enzyme weight, while the errors associated with the solution volume measurements and colorimetric readings were <0.1%. A reference curve was also obtained providing the amount of reducing matter vs the contact time: it was a straight line passing through the origin with slope 1.43. This curve was used to quantify the terminal reducing groups.

2.3. Enzymes

The following enzymes were used at the concentration of 3.2 mg/100 ml, with the exception of β-amylase; in most cases water was preferred to the buffer because no pH alteration was noticed. Egg white lysozyme, Calbiochem; *Carica papaya* papain, Calbiochem; wheat germ lipase, Sigma; *Clostridium histolyticum* collagenase, Sigma; porcine pancreas α-amylase, Sigma; barley malt α-amylase, Sigma; and sweet potato β-amylase, Sigma; at nearly neutral pH values and 25 and 37°C.

2.4. Polysaccharides

The following fully water-soluble pullulans were studied: pullulan from *A. pullulans*, supplied by Sigma; a 500 KDa pullulan, and a 100 KDa pullulan, both supplied by the Department of Biology and Plant Pathology, University of Bari.

To prepare oxypullulan, pullulan (0.5 g) was dissolved in water (25 ml), then the catalyst, the stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo®, Aldrich, Milan) (6 mg) with NaBr (0.2 g) and $NaClO_4$ (10 g, 4%) were added at 4–8°C. The pH value was kept constant with 0.5 M NaOH (ca. 4.5 ml); after 20 min, the solution was dialysed against water for 48 h and then freeze-dried.

The oxypullulan–chitosan complex was prepared by adding to a oxypullulan sodium salt solution (0.3 g in 25 ml water) a 1% Primex chitosan solution in acetic acid (30 ml) under stirring until flocculation was complete. The complex was extensively washed with water and freeze-dried.

Oxychitin was prepared according to Muzzarelli et al. 1999a, from chitin supplied by Primex, Norway.

The oxychitin–chitosan complex was prepared as follows: to a 1% oxychitin sodium salt solution a 1% Primex chitosan solution in acetic acid was slowly added under stirring until flocculation was complete (molar ratio 1). The complex was extensively washed with water and freeze-dried.

2.5. Prosthetic materials

The sand-blasted Ti–6Al–4V alloy was coated either with hydroxyapatite or with AP40 bioglass, by plasma spray, as previously described (Muzzarelli et al., 2001). They were further treated by immersion in an aqueous 1% chitosan acetate salt solution. Two different methods were then employed to stabilize and modulate coating stability and biodegradability:

1. formation of polyelectrolyte complexes with 6-oxychitin: chitosan-coated plates were immersed for 10 min in oxychitin solution (0.2 g in 100 ml water), and kept in a dryer at room temperature for 24 h,
2. formation of amide functions between chitosan and 6-oxychitin, samples from 1 were immersed in an aqueous solution containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.2 g in 4 ml water) for 2 h at 4°C to promote the formation of amide bonds between chitosan and oxychitin.

These procedures yielded differently coated plates, indicated as hydroxyapatite, hydroxyapatite–chitosan–oxychitin, hydroxyapatite–chitosan–oxychitin amide, AP40 bioglass, AP40-chitosan–oxychitin amide, from which nails 2 mm in diameter and 4 mm in length were obtained.

2.6. Animal model

Of 36 female Sprague Dawley rats (Charles River, Calco, Lecco, Italy) aged 10 months (400 ± 20 g b.w.), 18 were bilaterally ovariectomised and 18 were left intact (Control). Four months later, the left femoral condyle of all animals was exposed by longitudinal skin incision under general anaesthesia (i.m. injection of a mixture of 87 mg/kg Ketamine; Ketavet, Farmaceutici Gellini, Aprilia, Latina, Italy and 13 mg/kg xylazine; Rompun, Bayer Italia SpA, Italy.

One nail of each material was transversally implanted in three control and three ovariectomised animals to obtain six groups of implanted control rats and six of implanted ovariectomised ones. Two months after surgery the animals were pharmacologically euthanized (Tanax, Hoechst, Frankfurt am Main, Germany) and the femurs removed for histological examination.

3. Results and discussion

3.1. Pullulans

Pullulans (25 mg) were dissolved in water (50 ml), mixed with the enzyme solution (25 ml) and kept at the desired temperature for the desired time. The amount of reducing end groups formed as a result of enzymatic degradation was determined using ferricyanide, and the differences in ferricyanide absorbance, ΔAbs, corresponding to the amounts of the reducing end groups were plotted as a function of the enzyme contact time.

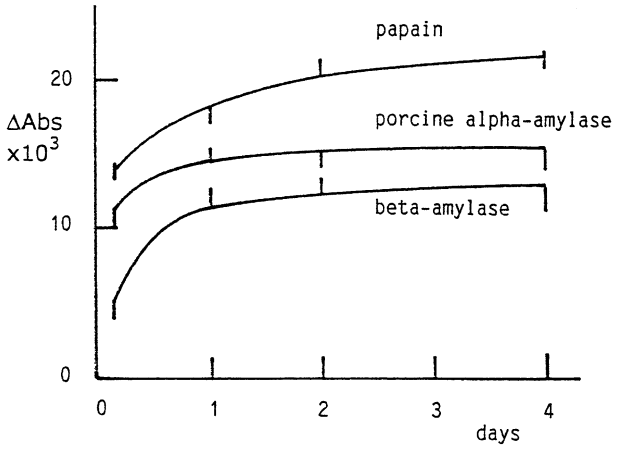

Fig. 1. Reducing activity increase due to the enzymatic hydrolysis of the Sigma pullulan in acetate buffer solution, expressed as net absorbance as a function of time.

All pullulans were hydrolysed by the tested enzymes, namely papain, α-amylases of animal and plant origin and β-amylase. The Sigma pullulan showed maximum susceptibility to papain; amylases indicated a ΔAbs close to 0.015 after one day. During the next few days, no significant increases of ΔAbs were noticed, as shown in Fig. 1. Solutions of Sigma pullulan (10%) were observed in the time period of 270 min at 25 and 37°C in the presence of porcine pancreas amylase (55 mg/ml). Fig. 2 shows the linear viscosity decrement that, after 24 h at 25°C, reached 13 mPa. This linear behavior is quite different from the observed logarithmic trends in the unspecific enzymatic degradation of chitosan and methylpyrrolidinone chitosan (Muzzarelli et al., 1992, 1995), and indicates a slow hydrolytic process operated by the animal amylase.

The 500 KDa pullulan was particularly susceptible to the pancreatic amylase, with a ΔAbs of 0.541, while for the plant amylases ΔAbs was approximately 0.043 (see Fig. 3). The 100 KDa pullulan was also extensively hydrolysed

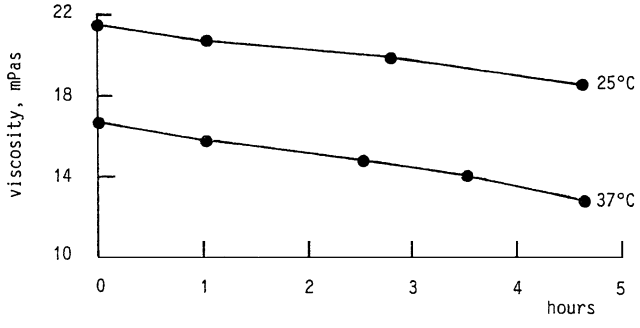

Fig. 2. Viscometric curves obtained for Sigma pullulan in the presence of porcine pancreas α-amylase at 25 and 37°C.

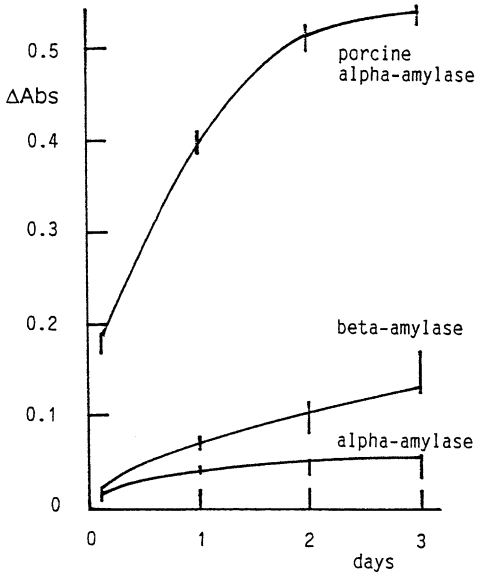

Fig. 3. Reducing activity increase due to the enzymatic hydrolysis of the 500 KDa pullulan, expressed as net absorbance as a function of time.

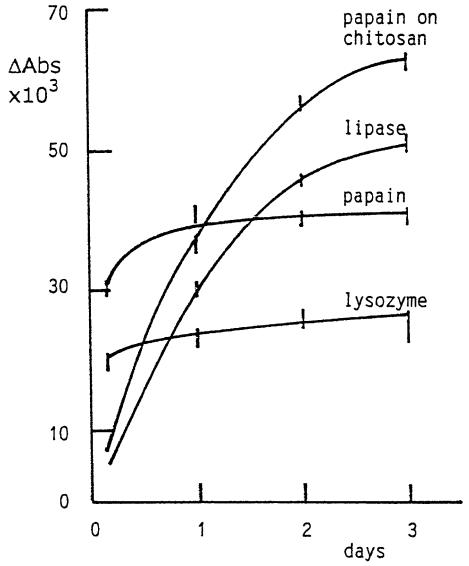

Fig. 5. Reducing activity increase due to the enzymatic hydrolysis of the oxychitin-chitosan complex, expressed as net absorbance as a function of time. A curve for solid chitosan powder (100–200 mesh) in suspension is included.

by animal amylase with ΔAbs 0.4 after three days, the ΔAbs being 0.06 per day in the absence of the enzyme (Fig. 4).

3.2. Oxypullulan–chitosan complex

The analytical data showed that the oxypullulan–chitosan complex was not a substrate for the enzymes tested, in spite of the fact that its chitosan component, when alone, was enzymatically degradable. This was a remarkable point of difference compared to the oxychitin–chitosan complex that might be justified by different structures of the polysaccharide complexes, presently under study.

3.3. Oxychitin–chitosan complex

The susceptibility of the oxychitin–chitosan complex to the selected enzymes was examined in comparison to chitosan as such (100–200 mesh powder) and oxychitin alone. The results showed that the oxychitin–chitosan complex is susceptible to depolymerization by lysozyme, papain and lipase (Fig. 5). In particular, the depolymerization by lysozyme is comparable to that of α-chitin by lysozyme, reported by Kurita et al. (2000) even though somewhat lower. Lipase and papain are more effective than lysozyme, nevertheless the solid chitosan powder in suspension is a better substrate than the oxychitin–chitosan complex for papain. The other enzymes listed under 2.3 were ineffective.

When lysozyme was tested with water-soluble oxychitin sodium salt, the initial activity value was strongly depressed within 24 h, indicative of important interferences and enzyme activity loss. With oxychitin, lipase did not loose much activity, while the papain curve matched the one for the oxychitin–chitosan complex as substrate. It would seem that papain was scarcely affected by oxychitin Na salt that, on the contrary, tended to coagulate some other proteins, as indicated by simple protein flocculation tests with oxychitin Na salt.

The high hydrophilicity of the oxychitin–chitosan complex and its swelling when brought in contact with aqueous media led to a significant degree of enzymatic susceptibility, notwithstanding the heterogeneous reaction conditions. Compared to chitosan, the oxychitin–chitosan complex contains of course a lower density of *N*-acetylglucosamine units crucial for a substrate to be recognized by lysozyme.

In spite of this limitation, the oxychitin–chitosan complex appears to be biodegradable by lysozyme and lipase, two enzymes widely occurring in wounded and

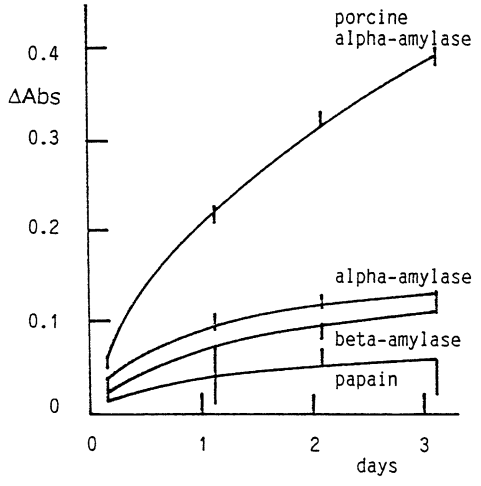

Fig. 4. Reducing activity increase due to the enzymatic hydrolysis of the 100 KDa pullulan, expressed as net absorbance as a function of time.

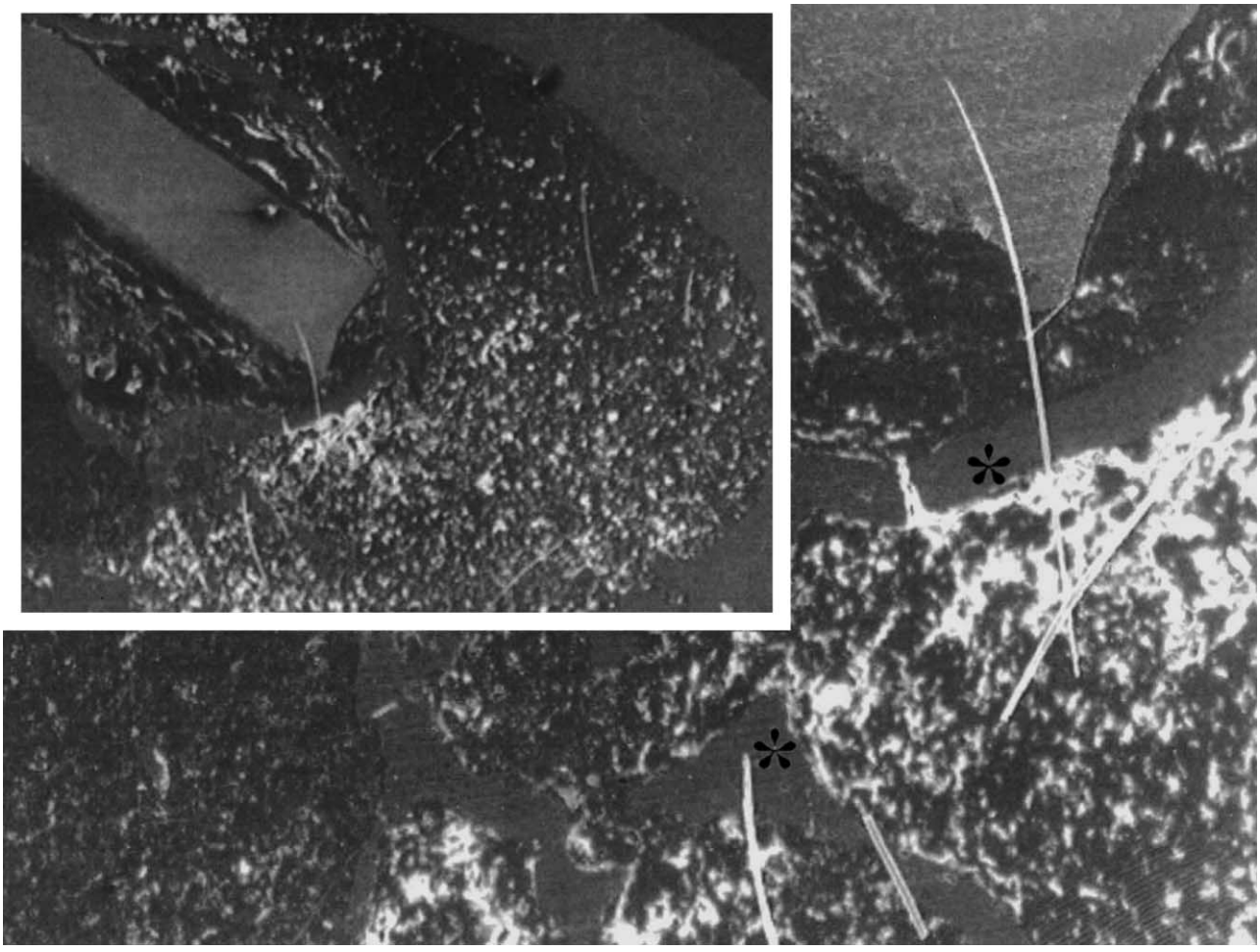

Fig. 6. Scanning electron micrograph of hydroxyapatite-coated implant in ovariectomized rat. The hydroxyapatite–chitosan–oxychitin association exerts a strong osteoinductive effect (*). Inset: low-magnification of hydroxyapatite–chitosan–oxychitin implant in ovariectomized rat.

healthy tissues; therefore the present results, including those for α-amylase, appear to be of relevance to the use of the oxychitin–chitosan complex in wound healing and drug delivery.

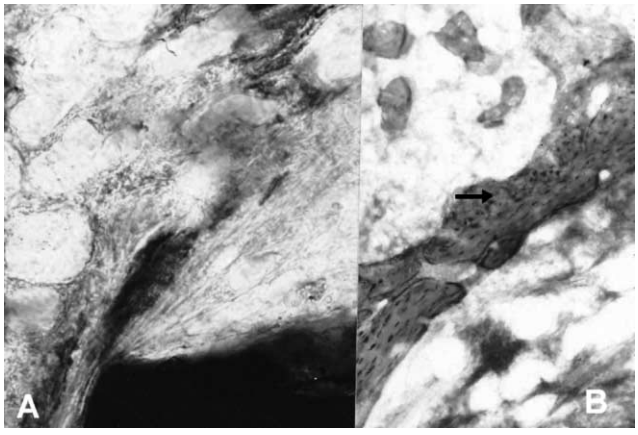

Fig. 7. Light micrograph of hydroxyapatite implant in ovariectomized rats. (A) note the good implant-bone integration. (B) The oxychitin overcoat potentiates the neo-osteogenic effect (→).

3.4. Biochemical activity and resorption of the oxychitin–chitosan complex

In control rats the morphological data showed a satisfactory reaction of the bone in contact with the control nails as well as with those coated with oxychitin–chitosan complex and oxychitin–chitosan polyamide. In general, the osteoreactive response was weaker in rats bearing oxychitin–chitosan complex implants than in control rats.

Under these experimental conditions, both chitosan–oxychitin associations seemed to promote a better structural response in terms of bone integration than the chitosan–coated AP40 biological glass. (Fig. 6). A greater amount of new bone tissue at the bone-prosthesis interface with oxychitin–chitosan complex, suggesting a greater osteoinductive activity of this complex compared with that of the covalent association oxychitin–chitosan polyamide (Figs. 7 and 8). Elemental analysis did not evidence any adverse effect on mineralization, and showed that chitosan (particularly oxychitin–chitosan complex) enhanced osteoinduction more than mineralization.

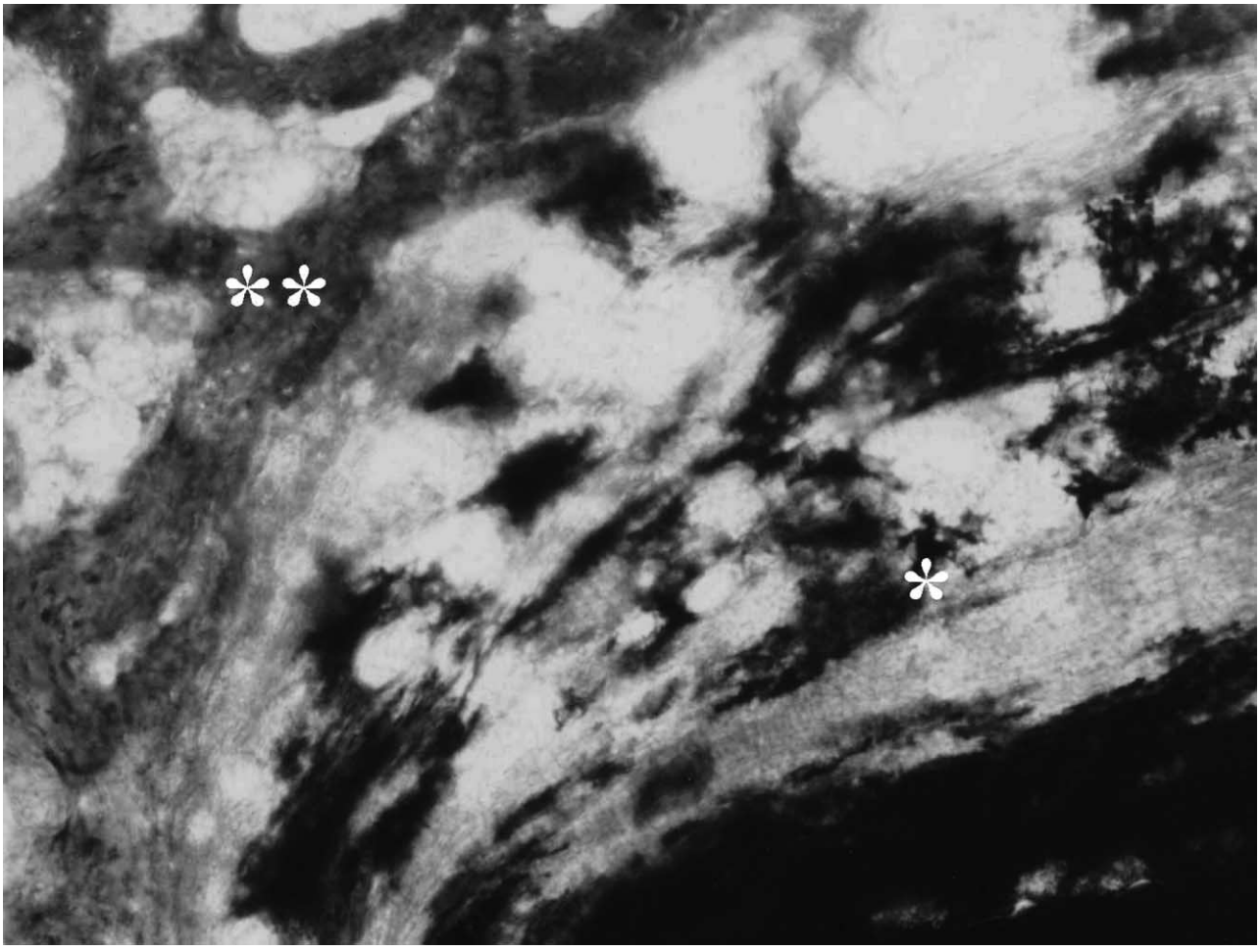

Fig. 8. Light micrograph of implant in ovariectomized rat. The hydroxyapatite–chitosanss–oxychitin polyamide overcoat enhances osteointegration (*) and new bone (* *) formation as well.

4. Conclusions

The colorimetric and viscosimetric data indicate that pullulans and the oxychitin–chitosan complexes are susceptible to unspecific hydrolytic action by a range of animal and plant hydrolases, that in most cases act slowly but effectively enough to depolymerize the insoluble polysaccharides complexes here examined. While pullulans and chitosans are promptly degraded by a number of hydrolases, the oxychitin–chitosan complex seems suitable for the modulation of the biodegradation process in vivo.

The oxychitin–chitosan complex was seen to induce significantly better interactions at the interface between bone tissue and prosthetic materials and to counteract the oxidative stress induced by ceramics and metals. It is however evident, at least from a morphostructural standpoint, that hydroxyapatite and AP40 bioglass alone are insufficient to induce osteointegration and osteoconductivity, and that these activities are crucially connected with the presence of chitosans (Mattioli-Belmonte et al., 1999; Yamaguchi et al., 2001).

The oxypullulan–chitosan complex does not seem prone to hydrolytic depolymerization under the conditions studied.

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