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Preparation and characterization of poly(ethylene glycol)-crosslinked reacetylated chitosans

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Abstract

Poly(ethylene glycol) (PEG) dialdehyde diethyl acetals of different molecular sizes were synthesized and used to generate in situ PEG dialdehydes for the crosslinking of partially reacetylated chitosan via Schiff reaction and hydrogenation of the aldimines. The water-soluble products obtained were instrumentally characterized. Upon freeze-drying, they aggregated to yield insoluble soft and spongy biomaterials, that swelled immediately upon contact with water. When exposed to papain and lipase, at physiological pH values, progressive dissolution of the biomaterials was observed, but no dissolution took place with lysozyme, collagenase and amylase. They were found to be biocompatible towards Caco-2 cells. These crosslinked partially acetylated chitosans seem suitable for medical items when prompt resorption is sought.

Keywords: Chitosan; Polyethylene glycol dialdehydes; Lipase; Papain

1. Introduction

Poly(ethylene glycols) (PEGs) are water-soluble amphipatic oligomers frequently used for biomedical applications, as hydrophilic galenic excipients endowed of biocompatibility and low biodegradability. Ointments, suppositories, tablets, injections preparation and capsules are formulated with PEGs. Similarly, PEGs are suitable for cosmetic preparations, ranging from lipsticks to hair sprays (Uglea & Dumitriu-Medvichi, 1994).

Various studies have been published on the modification of chitin and chitosan with high molecular weight PEGs. Sugimoto, Morimoto, Sashiwa, Saimoto and Shigemasa (1998) prepared chitosan–PEG hybrids and acetylated them to produce chitin–PEG hybrids: for this purpose chitosan was modified with PEG-monoaldehydes of various molecular weights under reducing conditions. Some of those derivatives were soluble in saline (PBS, pH 7.2). Similarly, monomethoxy polyethylene glycol aldehyde was used to modify chitosan by Mo, Aiba, Wang, Hayashi and Xu (1998), while Tokura et al. (1998) used PEG biscarboxyl chloride to crosslink chitosan. PEG-grafted liposomes containing 1,2-distearoyl glycerol phosphocholine were studied by Mobed and Chang (1998). Kim, Lee and Cho (1995) studied polymer networks composed of chitin and PEG, and Bentley, Roberts and Harris (1998) studied chitosan gels.

Grafting PEG onto chitosan is considered a convenient approach to water-soluble chitosan derivatives, to be used, for instance, as carriers of anticancer drugs. Nevertheless, in spite of their hydrosolubility, the PEG-grafted chitosans, obtained with the use of carbodiimide, were found to aggregate spontaneously in aqueous solutions, due to hydrogen bond formation by unmodified glucosamine units (Ouchi, Nishizawa & Ohya, 1998). PEG-modified chitosans have also been prepared in our laboratory via reductive alkylation by PEG monoaldehydes: the freely water-soluble products obtained reaggregated within a few weeks thus becoming insoluble.

Certain medical applications of chitin require initial insolubility accompanied by a defined biodegradability of the polymer. This is the case, for instance, of wound dressings suitable to prevent tissue adhesion in internal surgery. These materials should ideally be insoluble when positioned in the surgical wound, and undergo progressive bioerosion leading to complete resorption, as soon as they are no longer needed. Therefore, they should be enzymatically degradable (Jollès & Muzzarelli, 1999).

Highly hydrosoluble chitosans crosslinked to various extents should offer the possibility to modulate the

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biodegradability, and to provide medical items endowed with finely tuned biodegradability depending on the applications foreseen. For this purpose, partially reacetylated chitosan, that is fully water soluble at physiological pH values, and should therefore provide the most hydrophilic materials, was deemed here to be a suitable starting biopolymer.

Glutaraldehyde has been frequently used to crosslink chitosan, since the first report on chitosan–glutaraldehyde gels (Muzzarelli, Barontini & Rocchetti, 1976) and subsequent application for the immobilization of a variety of enzymes (Muzzarelli, 1980) and mammalian cells on chitosan (Kawase, Michibayashi, Nakashima, Kurikawa, Yagi & Mizaoguchi, 1997), as well as for drug release agents (Giunchedi, Genta, Conti, Muzzarelli & Conke 1998). Nevertheless, glutaraldehyde contains complicated chemical species (Muzzarelli, 1977) of documented cytotoxic nature; therefore, other dialdehydes and polyaldehydes have been proposed (see for instance, Crescenzi & De Angelis, 1997; Paradossi, Cavalieri & Crescenzi, 1997; Paradossi, Chiessi, Cavalieri, Moscone & Crescenzi, 1997).

The present work was aimed at introducing a certain degree of crosslinking in partially reacetylated chitosan, via PEG-dialdehyde diethyl acetals, and modulating solubility and biodegradability.

2. Experimental

2.1. Chemicals

Polyethylene glycols and other reagents were supplied by Sigma, Milano. PEGs were thoroughly dehydrated before use to eliminate traces of moisture, in a rotavapor, by repeated evaporations from a mixture of benzene:methanol (6:4) and then from toluene. Oxalyl chloride was freshly distilled. Dimethylsulphoxide and triethylamine were distilled on CaH_2. Dichloromethane was distilled on P_2O_5.

2.2. Enzymes

The following enzymes were used: collagenase from *Clostridium histolyticum* (Sigma C9891), papain from *Carica papaya* (Calbiochem 5125), wheat germ lipase (Sigma L-3001), egg white lysozyme (Calbiochem 4403), α-amylase from human saliva (Sigma A-1031). Each enzyme was separately dissolved in ultrapure water (from an Elgastat still) to prepare solutions containing 4 μg enzyme per ml; for the biodegradability test, one piece of freeze-dried material (ca 10 mg) was suspended in the enzyme solution (50 ml) at 25°C and mild occasional stirring was applied. After the observation period, the undissolved material was recovered and freeze-dried to determine the percent of dissolution. The initial and final pH values were between 6.5 and 7.3.

2.3. Chitosans

Chitosan (5 g) was suspended in water (490 g) for 30 min, and glacial acetic acid (5 g) was added to dissolve it; methanol (214 g) was added, followed by 98% acetic anhydride (2.86 g). The mixture was kept under stirring for 24 h, then kept in dialysis against demineralized water for 60 h. At the NMR analysis, the water-soluble partially reacetylated chitosan showed degree of acetylation 0.60. This final degree of acetylation was reached in all cases regardless of the initial degree of acetylation, usually in the range 0.20–0.30, provided that these experimental conditions were observed.

2.4. Analytical methods

Infrared spectra were recorded with a Bruker IFS 25 spectrometer and NMR spectra with a Bruker AC 300 spectrometer. 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 CuK_α radiation ($\lambda = 0.154$ nm) was used.

2.5. Synthesis of the crosslinking agents

2.5.1. Synthesis of tetraethylene glycol dialdehyde

The method described by Mancuso, Hang and Sweon (1978) was followed. Oxalyl chloride (1 ml, 11.33 mmol) was dissolved with 25 ml of dichloromethane, the solution cooled at −60°C, and DMSO (1.6 ml, 22.66 mmol) in dichloromethane (5 ml) was slowly dropped into the flask while keeping the temperature at −60°C. After 2 min, tetraethylene glycol (1 g, 5.15 mmol) dissolved in dichloromethane (10 ml) was added dropwise, and stirring was continued for an additional 20 min. Triethylamine (50 mmol, 7 ml) was added slowly while maintaining −60°C for another 5 min. Finally, the temperature was allowed to rise to −20°C and the reaction mixture filtered to remove most of the triethylamine hydrochloride, the filtrate diluted to 60 ml with dichloromethane and extracted with water (4 × 10 ml). The collected aqueous phases were washed with dichloromethane (5 ml) and taken to dryness under vacuum (0.1 mm), obtaining 778 mg of the dialdehyde. The oxidation degree detected by NMR was 90%. To avoid the fast polymerization of the purified product, the aqueous solution of tetraethylene glycol dialdehyde, after washing with dichloromethane, was directly used for the reaction with chitosan.

2.5.2. Synthesis of PEG dialdehyde diethyl acetals (general procedure)

Potassium *tert*-butoxide (16 mmol) was added to a solution of glycol (2.5 mmol) in benzene (20 ml); after 1 h stirring under nitrogen, bromoacetaldehyde diethyl acetal (20 mmol) was added, and the reaction mixture warmed at 65°C. After 2 h, the mixture was cooled to room temperature; additional potassium tert-butoxide (7 mmol) was

added, and, after 30 min, bromoacetal (20 mmol) to complete the reaction after stirring for 2 more hours at 65°C. The consumption of the reagent was monitored by TLC (chloroform:methanol, 96:4). The reaction mixture was diluted with hexane (20 ml), left in the freezer overnight and centrifuged to eliminate most of the salts. The crude residue was purified by flash chromatography, yielding slightly yellow oils consisting of pure PEG dialdehyde diethyl acetals, which could be safely stored at 4°C. In the case of the longest PEGs, the following modifications were introduced: (1) 40 ml of benzene were used for the reaction mixture; and (2) the reaction time was prolonged to 8 h. In the case of diethylene glycol, THF was substituted for benzene as the solvent for the reaction, due to solubility problems.

2.5.3. Synthesis of tetraethylene glycol disuccinate

Tetraethylene glycol (1 g, 5.15 mmol) was dissolved with chloroform (60 ml), then pyridine (2.1 ml, 25.75 mmol) and succinic anhydride (2.57 g, 25.75 mmol) were added to the mixture and refluxed for 5 h. The solution was evaporated to dryness and the residue dissolved with $NaHCO_3$ (20 ml, 5%), then acidified to pH 1 with 6 N HCl. The acidic solution was extracted with chloroform (1×50 and 6×40 ml). The collected organic fractions, after dehydration with Na_2SO_4, gave 1.93 g of a viscous oil, yield 95%.

2.5.4. Synthesis of the tetraethylene glycol disuccinimidyl disuccinate

The tetraethylene glycol disuccinate described above (146 mg, 0.37 mmol) and *N*-hydroxy-succinimide (0.93 mmol) were dissolved with ethylacetate (3.5 ml), and *N*,*N*′-dicyclohexylcarbodiimide (168 mg, 0.81 mmol) was added at 0°C. After 1 h, the reaction mixture was cooled at −20°C and filtered. The solution was evaporated to dryness and the residue recovered with dichloromethane (3 ml) and kept in the freezer until all the excess succinimide precipitated. After filtration, the evaporated solution gave 205 mg of the product as a viscous oil, yield 94%. ^{1}H NMR ($CDCl_3$) δ 4.30 (t $COOCH_2$), 3.75–3.65 (m, CH_2CH_2O), 2.96–2.78 (m, $CO(CH_2)_2CO$, succinate and succinimide).

2.6. Crosslinking of partially reacetylated chitosan with PEG-dialdehydes

2.6.1. Method 1

An aqueous solution containing glycol dialdehyde (2 ml, 2.5%, 0.27 mmol) was added dropwise to an aqueous solution containing chitosan (40 ml, 0.5%, 0.54 mmol as free amine). The mixture was vigorously stirred at room temperature for 2 h. Sodium cyanoborohydride (0.72 mmol) dissolved in water (1.5 ml) was added slowly to the mixture, while the pH was constantly adjusted at 5–6 with 1 N HCl. After 24 h at room temperature under vigorous stirring, the viscous solution was diluted with water to double volume and dialysed until unreacted glycol and salts disappeared, upon monitoring by TLC, from the concentrated washing solutions (ca 70 h). Finally, the chitosan solution was freeze-dried.

2.6.2. Method 2

Glycol dialdehyde diethyl diacetals were used. A solution of glycol diacetal (8.5%, 0.2 mmol) in 1 N HCl was stirred at room temperature for 1 h and then added dropwise to a chitosan solution (0.5%, 0.41 mmol as free amine) while maintaining the pH at 5–6 with 1 N NaOH. After 2 h under vigorous stirring, the reaction was completed as described under Method 1.

2.6.3. Method 3

The procedure described under Method 2 was followed, except that, in this case, the acidic solution after the deprotection of the acetal with HCl was adjusted to pH 6 with 1 N NaOH, and the reduction step was carried out at pH 7.

2.6.4. Method 4

The method described by Aiba (1993) was followed. Tetraethylene glycol disuccinimidyl disuccinate (117.7 mg, 0.2 mmol) dissolved in water (4 ml) was added to a chitosan solution (0.5%, 0.41 mmol as free amine) adjusting the pH to 7 with small amounts of 0.1 N NaOH. After 24 h at room temperature under stirring, the rather viscous solution was diluted with water to double volume and dialysed against water; *N*-hydroxysuccinimide but no glycol was detected in the concentrated washing solutions by TLC. The IR spectrum of the freeze-dried compound showed a very small band at 1725 cm^{-1} probably due to the carboxyl function. In a subsequent experiment, the reaction mixture after dialysis was treated with $NaHCO_3$ and dialysed again; noticeable amounts of glycol diemisuccinate were detected in the outer solution. The IR spectrum of the freeze-dried product was superimposable to that of the starting chitosan. ^{13}C NMR(D_2O) spectra confirmed the above findings: in fact, while the first lyophilized product showed signals at 182.87 (COO), 72.09 (CH_2CH_2O), 70.89 ($COOCH_2\underline{CH}_2O$), 66.34 ($COO\underline{CH}_2CH_2$), 32.83 ($CO(CH_2)_2CO$) due to the presence of PEG succinate mixed with the typical chitosan pattern, the same mixture after dialysis at pH 8 showed only signals due to the plain chitosan.

3. Results and discussion

3.1. Preparations

The products described in the present work and listed in Table 1 were obtained by reductive alkylation of the free amino groups of chitosan with PEG-dialdehydes. In fact, attempts of conjugation via PEG diemisuccinate activated

Table 1
List of the PEG-crosslinked chitosans

Sample No	Reagent	Method	Chitosan amine/ reagent molar ratio
1a	1	1	2
2a	3	2	2
3a	2	2	2
4a	3	2	4
5a	4	2	2
6a	5	2	2
7a	3	3	4

with *N*-hydroxysuccinimide, according to Aiba (1993) failed, giving a mixture of diacid and chitosan (see Section 2.6.4.). Free PEG-dialdehydes polymerize very fast, whereas they can be stored safely as diethyl acetals at 4°C for a long time. Therefore, PEG-dialdehyde diethyl acetals were preferred as reagents for crosslinking partially reacetylated chitosan. The reagents prepared here for use on partially reacetylated chitosan are presented in Table 2.

Once freeze-dried, all the products were white spongy insoluble materials. Micrographs of samples, in Figs. 1–3, show that their textures are quite different. All of them were however highly hydrophilic and swelled upon contact with aqueous solutions.

At the X-ray diffraction analysis, the partially reacetylated chitosan was amorphous, as well as the PEG-chitosans. Sample 7 showed a series of peaks between 2θ 17 and 24°, all of them attributed to chitosan.

As for ^{13}C NMR (D_2O–H_2O) of the samples in solution before freeze-drying, in comparison to the starting reacetylated chitosan, signals at δ 71.88 (CH_2CH_2O) and 49.50 (NH–CH_2) appeared, due to the glycol moiety.

The infrared spectra showed the methylene bands at 2876 cm^{-1}. The C/N ratios indicated a linear relationship with the PEG chain length.

Table 2
List of the reagents used to crosslink partially reacetylated chitosan, and ^1H NMR ($CHCl_3$) data, (δ)

Reagent	PEG derivative	Method	Yield%
1	CHO–$CH_2(OCH_2CH_2)_2OCH_2CHO$ 9.66 (t, CHO, $J = 0.88$); 4.09 (d, $\underline{CH_2}CHO$, $J = 0.88$); 3.67–3.50 (m, 8H, CH_2CH_2O).	2.5.1	79.4
2	$(EtO)_2$–$CHCH_2(OCH_2CH_2)_2OCH_2CH(OEt)_2$ 4.63 (t, CH, $J = 5.16$); 3.68–3.62 (m, 8H, CH_2CH_2O); 3.58 (q, $\underline{CH_2}CH$, $J = 7$; d, $\underline{CH_2}CH$, $J = 5.16$); 1.21 (t, $\overline{CH_3}$, $J = 7$)	2.5.2	73
3	$(EtO)_2$–$CHCH_2(OCH_2CH_2)_4OCH_2CH(OEt)_2$ See 2; 3.68–3.62 (m, 16H, CH_2CH_2O)	2.5.2	68
4	$(EtO)_2$–$CHCH_2(OCH_2CH_2)_{22.3}OCH_2CH(OEt)_2$ see 2; 3.68–3.62 (m, 88H, CH_2CH_2O)	2.5.2	65
5	$(EtO)_2$–$CHCH_2(OCH_2CH_2)_{46.2}OCH_2CH(OEt)_2$ see 2; 3.68–3.62 (m, 184H, CH_2CH_2O)	2.5.2	63

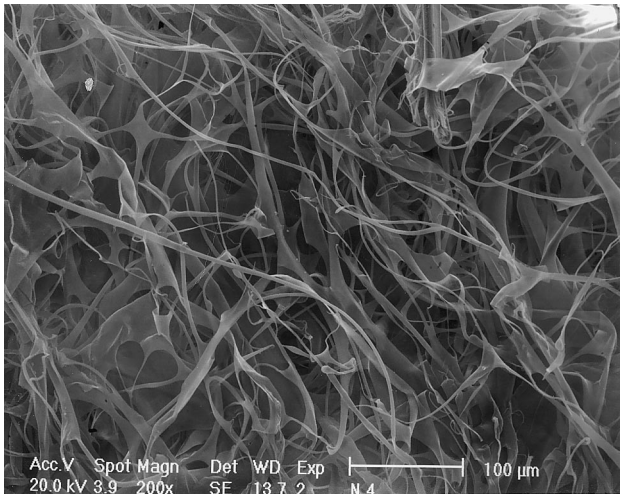

Fig. 1. Microphotograph of sample 3a (200×) showing the highly expanded texture of the crosslinked chitosan-PEG.

3.2. Enzymatic hydrolysis

When exposed to hydrolases, the PEG-chitosans were susceptible to the action of papain and lipase, whilst lysozyme was effective in one case only and amylase and collagenase were not at all. A domain that binds tightly to the solid polymeric substrate is present in some polysaccharide hydrolases, such as amylases (Wilson, Spezio, Irwin, Karplus & Taylor, 1995), and one could presume that such domain does no longer recognize the crosslinked chitosan.

In particular, samples 2a–6a were totally dissolved by papain within 1–2 h. The action of lipase was similarly very prompt, even though to a somewhat lower extent than papain. Lysozyme did not appreciably depolymerize the PEG-chitosans: this can be explained with the high degree of acetylation (0.60), that is far from the optimum (0.20) required by lysozyme.

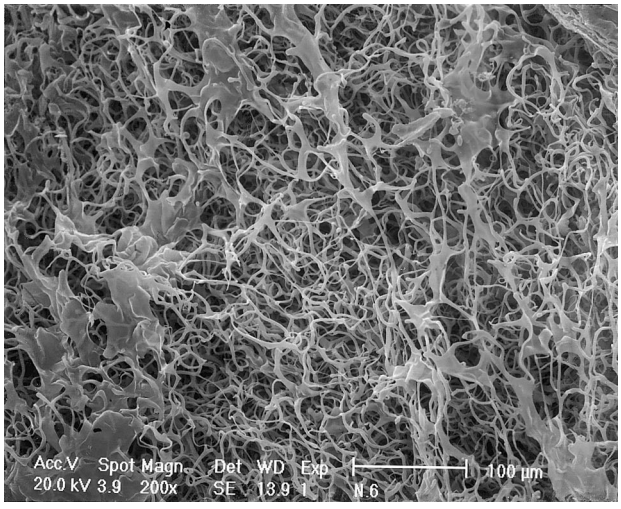

Fig. 2. Microphotograph of sample 5a (200×) showing the highly expanded texture of the crosslinked chitosan-PEG.

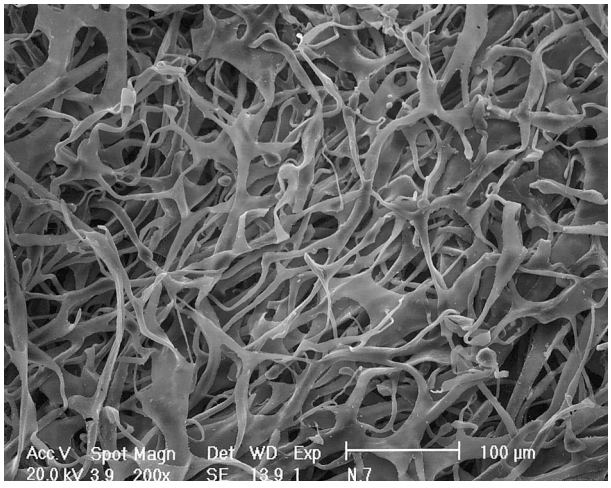

Fig. 3. Microphotograph of sample 6a (200×) showing the highly expanded texture of the crosslinked chitosan-PEG.

Of the three enzymes of importance for medical applications of these biopolymers (lipase, lysozyme and amylase), lipase only seems to play a significant role in the depolymerization of the PEG-chitosans. Therefore, if applied in vivo, the PEG-chitosans could be expected to be gradually dissolved by lipases present in most tissues.

The unspecific hydrolytic actions of papain and lipases are well-documented (Yalpani & Pantaleone, 1994). These enzymes of plant and animal origin respectively, can hydrolyse a variety of chitosans (Muzzarelli, Tomasetti & Ilari, 1994; Muzzarelli, Xia, Tomasetti & Ilari, 1995). It seems that they act on the carbohydrate chain of PEG-chitosans, irrespective of the length of the crosslinking agent. Of course, the progress of the hydrolytic reaction is slower than for unreacted chitosan (compare data by Muzzarelli et al., 1994; Muzzarelli et al., 1995), due to the more difficult access to the crosslinked carbohydrate chains; thus, the shorter PEG chains (sample 1a) delay the dissolution more markedly. It is also remarkable that sample 7a was not degraded by the lipase preparation. The data are summarized in Table 3.

3.3. Biocompatibility and cytotoxicity

Human colon carcinoma enterocyte-like cell line (Caco-2) (ATCC HTB37) was used for biocompatibility assessment, according to Rousset (1986). Cells were cultured at 37°C in 5% CO_2 humidified atmosphere in Dulbecco's modified Eagles minimum essential medium containing glucose (25 mM), L-glutamine (4 mM), sodium carbonate (3.7 mg ml^{-1}), nonessential aminoacids (1%) and supplemented with 10% calf serum, penicillin (50 U ml^{-1}) and streptomycin (100 mg l^{-1}). The test samples (4 × 4 mm) were immersed in 24-well cell culture plates using one well per sample and three wells per sample material. Three wells were for controls. Each well was filled with 0.25 ml of growth medium. The plates were incubated at 37°C in 5% CO_2 humidified atmosphere. After 4 h, the cells were seeded on each specimen (0.25 ml, 60 000 cells ml^{-1}). On day 9, the viability of the cells was determined by inverted microscopy using the Trypan Blue exclusion test. The Caco-2 cells exhibited compatibility for all crosslinked chitosan preparations. In general, cells remained viable and no cytotoxic effect was observed. Cytopatic focal aspects were evidenced mainly in samples 3a and 6a.

Table 3
Percent of the crosslinked biopolymer dissolved in water under the action of enzymes as a function of elapsed contact time, at 25°C

No	Time (h)	Papain	Lipase	Lysozyme	Amylase	Collagenase	
1a	7	50	25	0	0	0	
	24	100	100	0	0	0	
2a	0.5	50	50	0	0	0	No swelling
	1	100	100	0	0	0	
3a	0.5	100	75	0	0	0	
	24	100	100	0	0	0	
4a	0.5	90	75	0	0	0	No swelling
	24	100	100	0	0	0	
5a	1	100	90	0	0	0	
	24	100	90	0	0	0	
6a	1	100	–	0	0	0	
	24	100	100	0	0	0	
7a	24	90	0	0	0	0	

4. Conclusions

PEG-dialdehyde diethyl acetals are suitable compounds for the crosslinking of chitosan and partially reacetylated chitosan. Upon reduction of the chitosan aldimines, water soluble biomaterials are obtained, that however become insoluble upon freeze-drying, presumably due to aggregation phenomena that are often faced when handling chitosan derivatives. The aggregation seems to proceed slowly for some weeks during the storage of the freeze-dried biomaterials.

Crosslinking introduces high swelling capacity according to the length of the PEG used, and enhances hydrophilicity in the chitosans. They are therefore susceptible to papain and lipase, as a consequence of the unspecific hydrolytic action of these two enzymes towards chitosans, but are not found to be appreciably degraded by lysozyme, probably due to the high degree of acetylation of the partially reacetylated chitosan (Muzzarelli, 1992). Moreover, they are not degraded by collagenase and human α-amylase, because the substrate is no longer recognized.

Crosslinking of the highly water-soluble reacetylated chitosan with PEG-dialdehydes permits to obtain biomaterials of interest in the wound dressing area, because their rate of resorption can be modulated based on choice of PEG and crosslinking method. Even though it is presumed that human lysozyme would be scarcely active on these biomaterials, the ubiquitous human lipases are

expected to degrade these biomaterials in vivo, whilst collagenases do not seem to be active on these materials.

As an alternative to the use of glutaraldehyde, the present approach to the production of insoluble but highly hydrophilic chitosans can easily be extended to the area of material coating, where the surface modification of several biomaterials is desired in order to make them biocompatible.

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