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Alginate fibres modified with unhydrolysed and hydrolysed chitosans for wound dressings

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Abstract

A range of commercial chitosans were sourced, subjected to controlled acid hydrolysis, and their molecular size profiles and degrees of acetylation (DA) determined (pre- and post-hydrolysis) by High Performance Size Exclusion Chromatography and ^{1}H-NMR spectroscopy, respectively. Unhydrolysed and hydrolysed chitosans were subsequently utilised for modification of sodium alginate/alginic acid fibres (prepared using a range of different fibre spinning conditions), and levels of chitosan incorporated onto/into base alginate fibres were estimated by elemental analysis. Tensile properties (% elongation and tenacity) of resultant chitosan/alginate fibres were determined in order to assess their suitability for potential application in wound dressings. A broad range of chitosan contents (~0–6% w/w) and hydrolysed chitosan contents (~7–25% w/w) were obtained using a variety of alginate and chitosan starting materials. Modification of fibres with unhydrolysed chitosans generally resulted in a significant reduction in tenacity (and a reduction in % elongation if a water washing stage was not used), i.e. no increase in fibre strength was observed, implying that the unhydrolysed chitosan is more like a coating rather than penetrating/reinforcing the alginate fibre. Reduction of chitosan molecular weight had a positive effect on its ability to penetrate the alginate fibres, not only increasing fibre chitosan content, but also reinforcing fibre structure and thus enhancing tensile properties (compared with unhydrolysed chitosan/alginate fibres). Hydrolysed chitosan/alginate fibres demonstrated an antibacterial effect (in terms of bacterial reduction) with initial use, and had the ability to provide a slow release/leaching of antibacterially active components (presumably hydrolysed chitosan fragments). The overall aims of maximising chitosan content (in order to provide satisfactory antibacterial activity) whilst retaining desirable physical properties (i.e. satisfactory textile processing ability) were therefore achieved, with respect to the fibres having potential application as wound dressing materials.

Keywords: Chitosan; Hydrolysis; Alginate; Alginic acid; Fibre spinning; Tensile properties; Wound dressings

1. Introduction

Fibres have been extensively used in wound dressing applications because of their unique/advantageous properties, such as high surface area, softness, absorbency and ease of fabrication into many product forms. Fibres made from natural sources, especially polysaccharides, have been considered the most promising due to their excellent biocompatibility, non-toxicity, and potential bioactivity at the wound surface and beyond. Many commercial wound dressing products (woven and non-woven dressings, and hydrogels) are made from such natural polymers and their derivatives, the simplest being retention bandages, support and compression bandages, absorbents, gauzes, tulle dressings, and wound dressing pads produced from woven cellulose fibres (cotton and viscose) (British National Formulary, 2001; Kennedy, Knill, & Thorley, 2001; Kennedy, Paterson, Knill, & Lloyd, 1996; Lloyd, Kennedy, Methacanon, Paterson, & Knill, 1998).

Among the various fibrous and hydrogel products, alginate-based products are currently the most popular ones used in wound management, since they offer many advantages over traditional cotton and viscose gauzes (Horncastle, 1995; Qin, & Gilding, 1996). They are

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biocompatible and form a gel on absorption of wound exudate. This eliminates fibre entrapment in the wound, which is a major cause of patient trauma/discomfort during dressing removal. Such gelation prevents the wound surface from drying out, which is beneficial since a moist wound environment promotes healing and leads to a better cosmetic repair of the wound (Winter, 1962). Performance requirements for such gelled dressings (which often aim to replicate the inherent permeability/water content of natural skin) are obviously higher than mere absorbent coverings in order for the wound to remain moist during the contact period (which could be more than several days) (Thomas, 1990). Hence, it is also reported that alginate-based dressings have haemostatic properties and can enhance the rate of healing of skin wounds (Attwood, 1989; Jarvis, Galvin, Blair, & McCollum, 1987).

Commercial alginate-based dressings include Algisite® M (non-woven calcium alginate fibre, Smith and Nephew), Algosteril® (calcium alginate, Beiersdorf), Kaltocarb® (calcium alginate fibre, ConvaTec), Kaltogel® (calcium/sodium alginate gelling fibre, ConvaTec), Kaltostat® (calcium alginate fibres in non-woven pads, ConvaTec), Melgisorb® (calcium/sodium alginate gelling fibre, Molnlycke), Seasorb® (calcium/sodium alginate gelling fibre, Coloplast), Sorbalgon® (calcium alginate, Hartman), and Sorbsan® (calcium alginate fibres in non-woven pads, Maersk) (British National Formulary, 2001; Kennedy et al, 2001). Consequently there are numerous patents detailing the production of alginate fibres and dressings (Barikosky, 1999; Fenton, Keys, & Mahoney, 1998; Griffiths, & Mahoney, 1997; Horsler, 2000; Kershaw & Mahoney, 1999; Mahoney, & Howells, 1998; Mahoney, Pritchard, Howells, & Griffiths, 1999; Mahoney & Walker, 1999; Qin & Gilding, 2000; Thompson, 1996; Tong, 1985).

Another type of natural polysaccharide of interest with respect to wound management products is chitin, and its partially deacetylated derivative, chitosan. The presence of chitin/chitosan in a dressing is reported to promote fibroblast growth and affect macrophage activity, which accelerates the wound healing process (Balassa & Prudden, 1978; Hon, 1996; Mattioli-Belmonte, Muzzarelli, & Muzzarelli, 1997; Muzzarelli, Biagini, Damadei, Pugnaloni, & Da Lio, 1989; Muzzarelli, Mattioli-Belmonte, Pugnaloni, & Biagini, 1999; Technical textiles, 1995). Chitosans are biocompatible (since their biodegradation products are natural metabolites), and are used in a wide variety of commercial application areas, such as cosmetics, haemostatic agents, drug delivery vehicles, wound dressings, etc (Berscht, Nies, Liebendorfer, & Kreuter, 1995; Pittermann, Horner, & Wachter, 1997; Reports Group, Technical Insights, 1989; Skjåk-Bræk, Anthonsen, & Sandford, 1989). Although chitosan can be produced in powder, film, bead, fibre and fabric forms (Qin, & Agboh, 1998; Qin, Agboh, Wang, & Gilding, 1997), products made from pure chitosan fibres have not been commercially viable due to the high processing costs involved (deproteination, demineralisation and deacetylation processes are required to produce chitosan materials of adequate purity) and the availability of such purified material is still insufficient for large industrial scale fibre production. Poor textile processing properties of resulting fibres has also been a major problem.

The chemical structures of sodium alginate/alginic acid and chitin/chitosan are displayed in Fig. 1 (Collins, 1998). Alginic acid is obtained from the cell walls of brown algae (Phaeophyta) such as the seaweeds *Laminaria* sp. and *Ascophyllum* sp (Clare, 1993). It is a linear block copolymer composed of uronic acid residues, namely β-D-mannuronic and α-L-guluronic acid, linked by (1 → 4)-linkages. The distribution of the uronic acids along the chain is non-random and involves relatively long sequences of each uronic acid. In the presence of divalent cations, such as calcium, alginate gels can be formed due to ionic cross-linking via calcium bridges between L-guluronic acid residues on adjacent chains (McDowell, 1974). Chitin is a naturally occurring polysaccharide found in the outer shell of crustaceans, and is composed of 2-acetamido-2-deoxy-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine residues), linked by (1 → 4)-linkages. Chitosan is partially deacetylated chitin and is therefore composed of 2-amino-2-deoxy-β-D-glucopyranose (D-glucosamine) and *N*-acetyl-D-glucosamine residues.

Alginate fibres are generally prepared by injecting a solution of water-soluble alginate (usually sodium alginate)

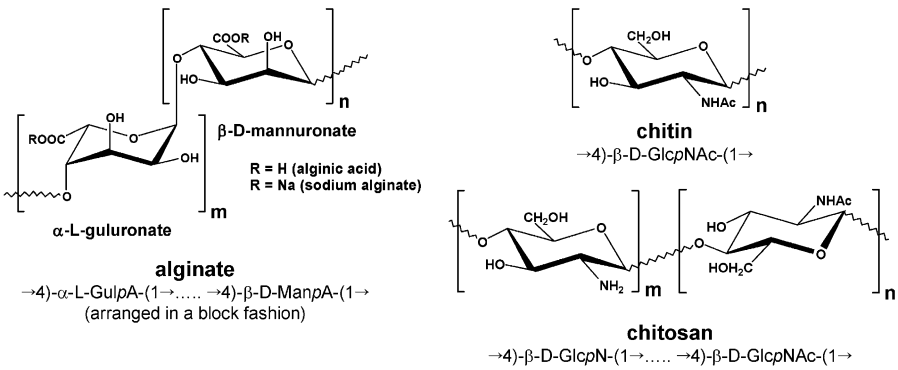

Fig. 1. Structures of sodium alginate/alginic acid and chitin/chitosan.

into a bath containing an acidic solution and/or calcium salt solution to produce the corresponding alginic acid and/or calcium alginate fibres, respectively, which can be used to produce yarns and fabrics for medical applications (Chen, Wells, & Woods, 2001; Miraftab, Qiao, Kennedy, Anand, & Collyer, 2001; Miraftab, Qiao, Kennedy, Groocock, & Anand, 2002; Qin et al., 1997). Many of chitosans properties rely on its cationic nature, which allows it to interact with negatively charged biomolecules such as proteins, anionic polysaccharides and nucleic acids, many of which are located in skin. Therefore, under certain conditions, alginate and chitosan have opposite and therefore mutually attractive charges, making the use of mixed dope solutions of suitable concentrations unfeasible, with respect to chitosan/alginate fibre production, since rapid coagulation/gelation of dope solution can occur.

Chitosan has been used to coat calcium alginate filaments (utilising the cationic interaction of the chitosan with the anionic nature of the alginate to produce a tight interaction) (Tamura, Tsuruta, & Tokura, 2002). However, due to its high molecular weight the chitosan must be used in very low concentrations, as precipitation occurs in the presence of calcium ions, resulting in very low levels of chitosan incorporation into the fibres (<0.2% w/w). Problems in the direct production of chitosan/alginate fibres have been overcome using a variety of different approaches. Alginate and chitosan fibres have been separately produced and subsequently blended, and chitosan has been utilised as the insolubilising cation for production of an alginate fibre (Cole & Nelson, 1993; Pandit, 1998).

The approach adopted for the production of fibres presented in this paper was the use of an initial core fibre produced using one of the polysaccharides, and subsequently applying the other polysaccharide by absorption into/coating onto this core fibre. The obvious route was to use an alginate core fibre (since the required methodologies for the production of fibres with suitable physicochemical characteristics are well known). The aim of the investigations was to produce fibres that combine the biomedical properties of both alginate and chitosan, and that have good textile processing ability and relatively low production costs. Alginate would essentially manage excess liquid/exudate and chitosan would provide antibacterial, haemostatic

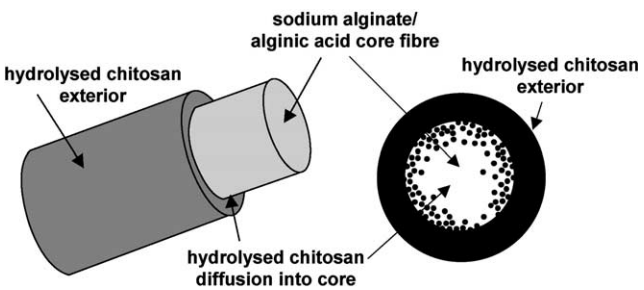

Fig. 2. Representation of an alginate/chitosan fibre showing the absorption of chitosan onto/into the base alginate fibre.

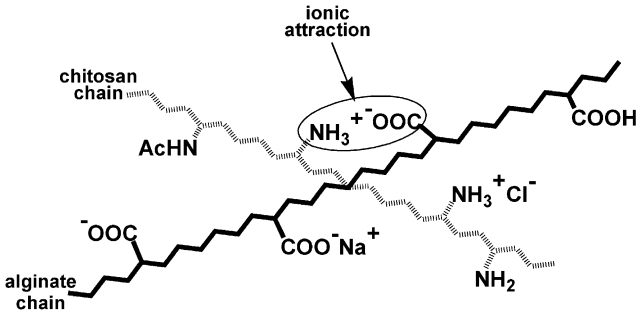

Fig. 3. Potential ionic interactions between sodium alginate/alginic acid fibres treated with chitosan.

and wound healing properties. It was predicted that use of hydrolysed chitosans should result in higher levels of chitosan incorporation, since fragments (molecules of lower molecular size than the parent unhydrolysed molecules) should be able to more easily penetrate the base alginate fibre structure (as illustrated in Fig. 2). It was also hoped that penetration of hydrolysed chitosan fragments into base alginate fibres would result in some reinforcement and thus increase/enhance tensile properties (perhaps via ionic interactions as illustrated in Fig. 3).

The comparative analysis of a range of commercial chitosans and their hydrolysates are therefore reported, along with the production and analysis of a range of chitosan/alginate fibres, produced by treating freshly extruded alginate fibres with unhydrolysed or hydrolysed chitosan solutions. The tensile properties (% elongation and tenacity) of the produced fibres were also evaluated in order to evaluate their suitability for potential use in wound dressings applications.

2. Materials and methods

2.1. Materials

A number of different (in terms of viscosity, molecular weight profile, composition, etc) sodium alginate and chitosan starting materials were obtained (as detailed in Table 1). D-glucosamine hydrochloride (GlcN.HCl, Sigma-Aldrich) and *N*-acetyl-D-glucosamine (GlcNAc, Sigma-Aldrich) were used (as controls) to treat selected sodium alginate/alginic acid fibres. Non-carbohydrate materials utilised included acetic acid (AcOH, CH_3COOH, >99%, Fisher Chemicals), acetone (Fisher Chemicals), calcium chloride ($CaCl_2$, Fisher Chemicals), deuterium oxide (D_2O, 99.9%, SigmaAldrich), hydrochloric acid (HCl, 37%, Riedel-de Haën®), sodium acetate trihydrate ($NaOAc.3H_2O$, Acros Organics), sodium chloride (NaCl, Fisher Chemicals) and trifluoroacetic acid (TFA, CF_3-COOH, 99%, Sigma-Aldrich). Shirlastain A dye mixture (SDL International) was used for fibre staining.

Table 1
Sodium alginate and chitosan materials obtained for fibre production

Code	Material	Product	Supplier	Viscosity (mPa.s, 1% soln)
A1	Sodium alginate	Protanal LF 10/60	Pronova	20–70
A2	Sodium alginate	Manucol® DH	ISP alginates	40–90
A3	Sodium alginate	Manugel® GMB	ISP alginates	110–270
C1	Chitosan HCl	Seacure CL 310	Pronova	200–800
C2	Chitosan	Seacure 443	Pronova	Unknown
C3	Chitosan	Kate 50–100	Kate Int.	50–100 (in AcOH)
C4	Chitosan	Type 222 (high viscosity)	France-chitine	200–2000 (in AcOH)
C5	Chitosan	Type 242 (high purity)	France-chitine	20–100 (in AcOH)

Information supplied by supplier.

2.2. Chitosan hydrolysis

Pre-dried chitosans (30.5 g) were dissolved in acetic acid solution (1% v/v, 757.5 ml, i.e. deionised water (750 ml) plus acetic acid (AcOH, 7.5 ml)). The solutions were stirred until viscous clear solutions were obtained (~3–4 h). Hydrochloric acid (HCl, 25 ml) was added to each solution and vigorous stirring was applied until homogenous solutions were obtained (~1/2–1 h). The resulting solutions (3.90% w/v, based on initial chitosan loading) were heated under reflux (for 8 h), cooled, filtered (to remove any insoluble material, and were ready for use for the treatment of sodium alginate/alginic acid fibres. Hydrolysed chitosan solutions of different volume and/or chitosan concentration were prepared by scaling/altering the amounts used accordingly.

Aliquots of hydrolysed chitosan solutions (~50–100 ml) were placed in CelluSep® T1 regenerated cellulose tubular membrane dialysis bags (flat width 55 mm, nominal molecular weight cut off—MWCO 3.5 kDa, Membrane Filtration Products, Inc, USA) and were dialysed against running water (for 48 h) to remove low molecular weight materials (especially salts). After dialysis, bag contents were quantitatively transferred into round bottom flasks with deionised water and were rotary evaporated to dryness (under reduced pressure at 50 °C). Resultant hydrolysates were dried to constant mass using a Mettler-Toledo HR73 Halogen Moisture Analyzer (standard drying mode, 80 °C) and stored over silica gel in a vacuum desiccator until required for analysis. A small quantity (~10 ml) of hydrolysed chitosan C3 dialysate was retained for anti-bacterial testing (as detailed in Section 2.10)

2.3. Chitosan molecular size profiling

The molecular size profiles of the dried unhydrolysed and hydrolysed chitosans were determined by High Performance Size Exclusion Chromatography (HPSEC) using Progel™-TSK PW_{XL} guard, G6000, G4000 and G2500 columns linked in series in a Knauer column oven and control unit (30 °C). The mobile phase was He degassed acetic acid (AcOH, 0.5 M) containing sodium acetate (NaOAc, 0.2 M), pumped at a flow rate of 0.5 ml/min using a Knauer HPLC pump 64. Chitosan samples and pullulan polysaccharides standards (M_W 853, 380, 186, 100, 48, 23.7, 12.2 and 5.8 kDa, Polymer Laboratories, UK) were dissolved overnight in system eluent (1 mg/ml, at 4 °C). All sample and standard solutions were filtered (using Titan nylon membrane filters, 0.20 μm) prior to loading into a Waters 712 WISP autoinjector (100 μl injections were analysed). Detection was performed using a Knauer differential refractometer (DRI) connected via a Dionex UI 20 Universal interface to a PC for system control using Dionex Chromeleon software (v. 6.11). Collected data was analysed using Polymer Laboratories PL Caliber Reanalysis software (v. 7.04).

2.4. Chitosan degree of acetylation (DA)

Dried unhydrolysed and hydrolysed chitosans were dissolved in deuterium oxide (D_2O, 20 mg/ml) containing trifluoroacetic acid (TFA, 5% v/v) overnight at ambient temperature. ^{1}H-NMR spectra of the resultant chitosan solutions were recorded at ambient temperature using a Brüker AMX 300 MHz NMR spectrometer. Resultant spectra were manipulated and areas of interest integrated using Brüker 1D WINNMR software. The degree of acetylation (DA) was calculated from the ratio of the normalised *N*-acetyl group proton peak area to the chitosan backbone proton area (Roberts, 1992).

2.5. Chitosan/alginate fibre production

Alginate/alginic acid fibres were produced by a conventional wet spinning technique using a multi-functional laboratory extruder (designed in-house and built by Howden Engineering Services, UK, illustrated in Fig. 4). Spinning dope solutions (sodium alginate, 1–6% w/v, depending on type and viscosity) were extruded under pressure through a spinneret (200 holes, 76 μm diameter) into a coagulation bath containing either hydrochloric acid (0.2 M) and/or calcium chloride (1–3% w/v) to afford the corresponding alginic acid and/or calcium alginate fibres. The resultant fibres/filaments were then drawn between a first and second

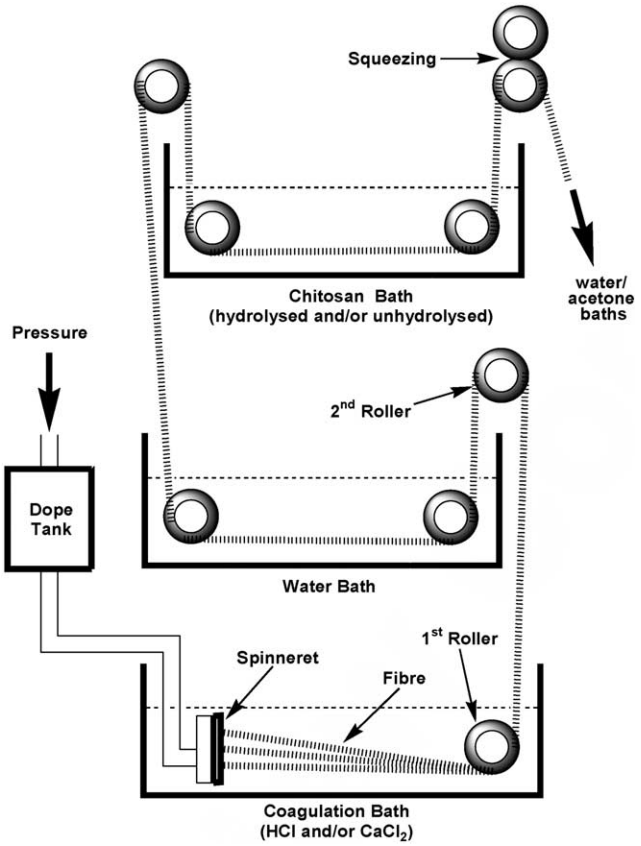

Fig. 4. Extrusion of chitosan/alginate fibres.

set of rollers (the relative speeds of which determine the draw ratio (DR), as detailed below) and were then passed through a water washing bath. The fibres were then squeezed (between rollers) to remove excess liquid, wound up, removed from the extruder and placed in a treatment bath containing unhydrolysed and/or hydrolysed chitosan (0–5% w/v in AcOH, 1% v/v) for 10 min, rinsed with deionised water and dried using acetone baths of increasing concentrations (50–100% v/v). Chitosan treatment, water washing and acetone drying can also be performed as continuous processes directly after production of initial fibres (as detailed in Fig. 4). Finally, fibres were separated by hand and conditioned (24 h at ambient temperature).

The Draw ratio (DR, or stretch ratio) is the ratio of the speeds of the first and second rollers used for production of the initial alginate fibre (as illustrated in Fig. 4), and provides an indication of the amount of stretching/molecular orientation the fibre has undergone. $DR = (S_2/S_1)$, where S_1 = speed of first roller and S_2 = speed of second roller (clearly roller speeds must be in the same units).

2.6. Chitosan/alginate fibre compositional analysis

Mean moisture contents of produced chitosan/alginate fibres (and starting materials) were determined by drying replicate fibre portions to constant mass using a Mettler Toledo HR73 Halogen Moisture Analyzer (standard drying mode, 80 °C). Mean ash contents of produced chitosan/alginate fibres (and starting materials) were determined using a standard ashing protocol (ashing replicate fibre portions to constant mass in a furnace, ~550 °C). CHN contents of produced chitosan/alginate fibres (and starting materials) were determined using an elemental analyser.

2.7. Fibre tensile properties

Elongation at break and tenacity of produced chitosan/alginate fibres were measured using a Textechno Fafegraph M single fibre tensile tester. The fibre sample is held between a set of clamps and a constant rate of extension is applied via a load cell and load vs elongation is recorded until point of failure. Values for % elongation and tenacity are then generated automatically. Elongation or extension describes the length by which a fibre extends when a load is applied. As the load is increased the elongation increases until the fibre breaks (under a specific load). The % elongation is determined from the ratio of the break length to the original length, % elongation $= (L_b/L_o) \times 100$, where L_b = break length and L_o = original length (clearly lengths must be in the same units). Tenacity defines fibre strength with respect to linear density, tenacity $= (B_L/D_L)$, where B_L = break load (N) and D_L = linear density (tex), which is the fibre weight in g/1000 m.

2.8. Microscopic analysis

Selected chitosan-alginate fibres were mounted on microscope slides using paraffin and cover slips, and were examined using a Nikon-Alphaphot-2 YS2-H optical transmission microscope. Image capture was performed using a Hitachi VK-C150E video link and fibre dimension measurements were performed using Image Pro software (v. 4.5).

2.9. Fibre staining

Selected chitosan-alginate fibres were incubated in a solution of Shirlastain A (1% w/w) for 2 min at ambient temperature. Fibres were then removed, repeatedly rinsed with deionised water and dried at room temperature. Shirlastain A is used to identify non-thermoplastic, natural, fibres such as cotton, wool and viscose. Different fibres pick up different shades of colour, depending on their molecular composition/structure.

2.10. Antibacterial testing

The antibacterial properties of selected chitosan/alginate fibres (and appropriate control fibres and solutions) were measured by shake flask testing (according to ASTM E2149-01 Standard Test Method for Determining

the Antimicrobial Activity of Immobilized Antimicrobial Agents under Dynamic Contact Conditions). Bacterial reduction (in terms of live bacterial cell count) following inoculation with *Staphylococcus aureus* (a well known Gram-positive bacterial inhabitant of colonised or infected wounds) was measured over a period of 0–24 h in:

(i) flasks containing fibres suspended in Sorensen's phosphate buffer (0.25 M KH_2PO4, adjusted to pH 7.2 ± 0.1 with dilute NaOH solution)
(ii) flasks in (i) having had their fibres removed after initial testing
(iii) flasks containing fresh buffer containing dried fibres removed from (i)

3. Results and discussion

3.1. Chitosan molecular size profiles

The molecular size profiles from HPSEC analysis of the unhydrolysed and hydrolysed chitosans (detailed in Table 1) are presented in Fig. 5. Retention times (R_T) for molecular weight calibration lines (10000, 5000, 1000, 500 and 100 kDa) were determined from linear regression analysis of the pullulan calibration curve ($\log M_W = -0.1539 R_T + 11.922, R = 0.9971, M_W$ = weight average molecular weight). The results of mathematical analysis of the molecular size profiles (using Polymer Laboratories PL Caliber Reanalysis software v. 7.04) are presented in Table 2.

Five molecular weight averages can be statistically calculated from molecular size profile data (M_n, M_w, M_z, M_{z+1} and M_v), and are defined by the equations detailed below. M_n is the number-average molecular weight and is simply the arithmetic mean, i.e. the sum of the molecular weights of all the molecules divided by the total number of molecules. M_w is the weight-averaged molecular weight, i.e. the weighted mean, and is the sum of the molecular weight squared of all the molecules divided by the total molecular weight of all the molecules. Increasing the weighting of the average gives rise to the z and $z+1$ averages. M_v is the viscosity average. w_i is the weight of i molecules with molecular weight M_i. N_i is the number of ith molecules with molecular weight M_i. d is the polydispersity. A d value close to unity indicates a narrow distribution.

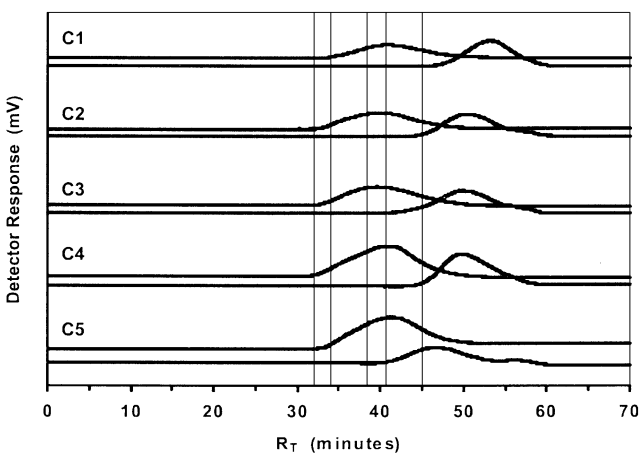

Fig. 5. Molecular size profiles of unhydrolysed and hydrolysed chitosans.

Table 2
Parameters obtained from analysis of unhydrolysed and hydrolysed chitosan molecular size profiles

Code	M_p (kDa)	M_n (kDa)	M_w (kDa)	M_z (kDa)	M_{z+1} (kDa)	M_v (kDa)	d (M_w/M_n)
Unhydrolysed chitosans:							
C1	447.7	133.6	698.4	1920.6	3350.0	577.1	5.2
C2	888.1	209.8	1214.9	2937.9	4345.8	1009.3	5.8
C3	697.1	144.5	993.2	2666.7	4338.4	813.6	6.9
C4	402.5	204.4	1033.8	3021.2	5154.0	841.3	5.1
C5	368.4	152.0	748.4	1948.1	3080.7	619.9	4.9
Hydrolysed chitosans:							
C1	5.5	4.1	8.4	16.4	36.8	7.5	2.0
C2	14.8	6.6	18.1	36.9	58.1	16.0	2.7
C3	17.7	7.3	32.3	121.2	256.0	25.8	4.4
C4	18.6	7.0	20.0	42.0	68.1	17.5	2.8
C5	55.3	24.1	74.0	162.9	256.0	64.0	3.1

$$M_n = \frac{\sum w_i}{\sum N_i} = \frac{\sum (N_i M_i)}{\sum N_i} \qquad M_{z+1} = \frac{\sum (N_i M_i^4)}{\sum (N_i M_i^3)}$$

$$M_w = \frac{\sum (w_i M_i)}{\sum w_i} = \frac{\sum (N_i M_i^2)}{\sum (N_i M_i)} \qquad M_v = \left[\frac{\sum (N_i M_i^{a+1})}{\sum (N_i M_i)} \right]^{1/a}$$

$$M_z = \frac{\sum (N_i M_i^3)}{\sum (N_i M_i^2)} \qquad d = \frac{M_w}{M_n}$$

Acid hydrolysis causes depolymerisation, resulting in formation of fragments with a range of molecular sizes. The size distribution of the chitosan fragments will have an effect on their incorporation into alginate fibres. Large molecules may not be able to penetrate the fibre and will therefore only interact with the fibre surface, whereas smaller fragments could partially penetrate the fibre network and become entangled. Even smaller fragments could fully penetrate the fibre network and become entangled, and very small fragments could easily penetrate the fibre network without any entanglement.

Chitosan C1 had the lowest initial M_n and M_w values and its hydrolysis consequently produced the lowest molecular size averages. Chitosans C2 and C4 had similar initial profiles and underwent similar degrees of hydrolysis. Chitosan C3 underwent less hydrolysis than chitosans C2 and C4, and had a significant proportion of higher molecular size material after hydrolysis, resulting in a much higher degree of polydispersity (d) after hydrolysis. Chitosan C5, although not having the highest initial molecular size averages, was the most resistant to hydrolysis, resulting in by far the highest molecular size averages after hydrolysis.

Table 3
Definition of useful terms (as applied to chitin/chitosan)

Term	Definition
Acetylation	The presence or addition of N-acetyl groups (i.e. n, or conversion from m to n, in Fig. 1)
Deacetylation	The absence or removal of *N*-acetyl group (i.e. m, or conversion from n to m, in Fig. 1)
Degree of acetylation (DA) or degree of substitution (DS)	Average number of acetyl groups per monosaccharide residue, i.e. DA of 0.1 means 1 out of every 10 GlcN residues acetylated (max. DA is 1, i.e. chitin).
Degree of deacetylation (DD)	Average number of free amino groups per monosaccharide residue, i.e. a DD of 0.1 means 9 out of every 10 GlcN residues acetylated (max. DD is 1, fully deacetylated).
% DA (% acetylation)	Number of acetyl groups present as a % of the total groups (assuming 0% acetylated, DA = 0, at start). Thus 20% acetylated is a DA of 0.2.
% DD (% deacetylation)	Number of acetyl groups removed as a % of the total groups (assuming 100% acetylated, DA = 1, at start). Thus 20% deacetylated is a DA of 0.8.

(DA + DD = 1, likewise % DA + % DD = 100%).

3.2. Degree of acetylation (DA) of unhydrolysed and hydrolysed chitosans

Comprehension of the terminology detailed in Table 3 is required in order to understand degrees of acetylation (DA), substitution (DS) and deacetylation (DD). The ^{1}H-NMR spectra of the analysed unhydrolysed and hydrolysed chitosans are composed of broad multiplets (rather than well resolved peaks) due to peak broadening as a result of viscosity (the ^{1}H-NMR spectra for unhydrolysed and hydrolysed chitosan C3 are presented in Fig. 6). The peak at ~1.8 ppm corresponds to *N*-acetyl methyl group hydrogen atoms, whilst the collection of peaks in the ~2.5–5.5 ppm region corresponds to chitosan backbone protons (i.e. single hydrogen atoms attached to the C_1–C_5 carbon atoms, and the two hydrogen atoms attached to the C_6 carbon atom) (Vårum, Rosenlund, & Smidsrød, 1989). Trifluoroacetic acid (TFA) moves hydroxyl protons to a higher chemical shift (>6 ppm, i.e. out of the area of interest).

The results of ^{1}H-NMR spectroscopic analysis of the unhydrolysed and hydrolysed chitosans are presented in Table 4. DA values were calculated from the ratio of the normalised acetyl group proton peak area to the chitosan backbone proton area (Roberts, 1992), determined by integration of respective peaks using Brüker 1D-WINNMR software and corrected to account for a single proton. DA values were converted to % DA by multiplying by 100, and to % DD by subtracting the % DA value from 100.

All unhydrolysed chitosans had determined %DD values within supplier's specifications. Chitosans C1, C2 and C4 underwent a similar degree of deacetylation after acid hydrolysis (~7%). Chitosan C5 underwent the lowest degree of deacetylation after acid hydrolysis (~4%), but had the lowest acetyl content to begin with (8%). Chitosan C3 underwent by far the highest degree of deacetylation after acid hydrolysis (~11%).

At dilute acid concentration the rates of depolymerisation and de-*N*-acetylation are similar, whereas at higher acid concentrations the depolymerisation rate becomes more dominant (Vårum, Ottøy, & Smidsrød, 2001). However, depolymerisation between two acetylated residues is several orders of magnitude faster than between two deacetylated residues (Vårum et al., 2001), which may explain the higher

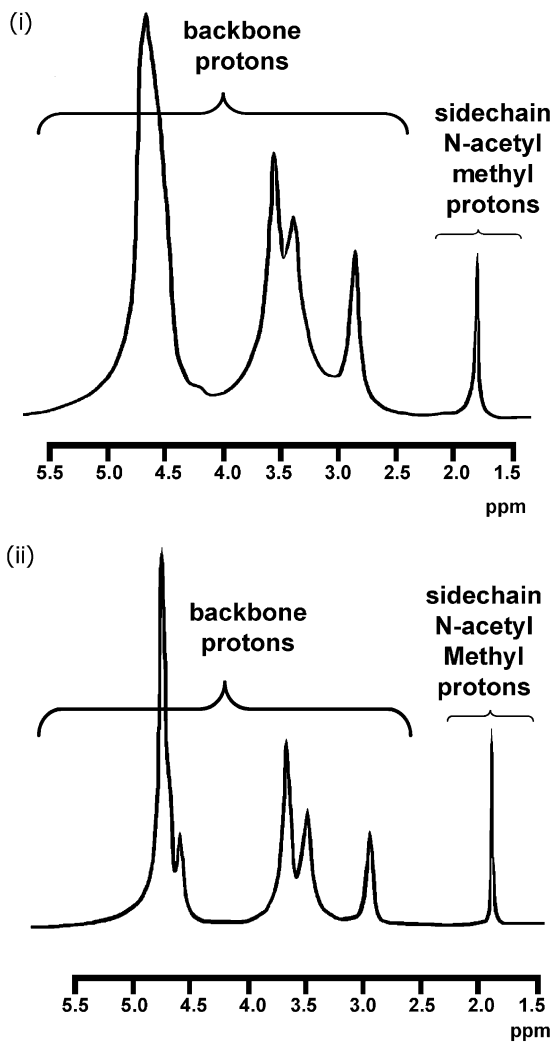

Fig. 6. ^{1}H-NMR spectra of (i) unhydrolysed, and (ii) hydrolysed chitosan C3.

Table 4
Degree of acetylation (DA) of unhydrolysed and hydrolysed chitosans (from integration of ^{1}H-NMR spectra)

Code	%DD (supplier spec.)	Unhydrolysed chitosan			Hydrolysed chitosan		
		DA	%DA	%DD	DA	%DA	%DD
C1	>70	0.28	28	72	0.21	21	79
C2	>60	0.33	33	67	0.26	26	74
C3	>80	0.16	16	84	0.05	5	95
C4	85–95	0.14	14	86	0.07	7	93
C5	>90	0.08	8	92	0.04	4	96

resistance of chitosan C5 to acid hydrolysis (it having by far the lowest initial DA).

3.3. Analysis of chitosan/alginate fibres

Variables associated with production of chitosan/alginate fibres included sodium alginate type, method of alginic acid fibre production and chitosan type, form (unhydrolysed or hydrolysed), and concentration. The appropriate alginic acid (100%) fibres (i.e. not treated with chitosan), and those treated with GlcN.HCl and GlcNAc, were also analysed as controls (Table 5). Information for selected fibres (alginate fibre and chitosan solution production parameters, and fibre analysis/testing results) is provided in Table 6 (using unhydrolysed chitosans) and Table 7 (using hydrolysed chitosans). The chitosan contents of fibres were determined from their nitrogen (N) contents (based on the nitrogen contents of the pure alginate fibres and the chitosan used for their production).

Unhydrolysed chitosan/alginate fibres (Table 6) produced from alginate A1 had significantly better physical properties than those fibres produced from both A2 and A3. Alginate A2 required a higher acid strength to produce suitable fibres (0.5 M compared with 0.2 M for A1 and A3), alginate A3 had to be used at a concentration of 4% w/w (since 6% w/w was too viscous), and both A2 and A3 fibres were produced at a draw ratio of 1.09 (compared with 1.18 for A1) due to the weakness of the fibres.

In general, use of a water washing stage resulted in the production of better fibres than without a water washing stage (in terms of both physical properties and chitosan contents). Treatment with unhydrolysed chitosans generally resulted in a reduction in tenacity (and a reduction in % elongation if a water washing stage was not used). All of the produced unhydrolysed chitosan/alginate fibres (Table 6) had inferior physicochemical properties to the parent alginate fibres (Table 5), suggesting that little or no penetration/reinforcement of the alginate fibre by the chitosan occurred.

Levels of hydrolysed chitosan incorporation using C1 and C2 chitosans were higher with A2 alginate base fibres, compared with A1 and A3 alginate base fibres, however those produced using A1 alginate base fibres had significantly better tensile properties (Table 7). Use of hydrolysed C2 chitosan resulted in significantly higher levels of incorporation than use of hydrolysed C1 chitosan, which is interesting since hydrolysed C1 chitosan had the smallest molecular size profile (see Fig. 5 and Table 2). Therefore, fragments over a certain size may be required in order to produce internal ionic cross-linking/reinforcement effects (as presented in Fig. 3). All of the base alginate fibres (A1-3) treated with hydrolysed C1 or C2 had significantly higher chitosan contents (~7–16% w/w) than fibres produced using unhydrolysed chitosans (~0–6% w/w).

Treatment of A1 and A3 base alginate fibres with increasing concentrations of hydrolysed C3 chitosan showed some interesting trends (Table 7). In the case of base A1 alginate fibres, treatment with increasing concentrations of hydrolysed C3 chitosan resulted in higher levels of chitosan incorporation into the base A1 alginate fibres. This was also paralleled by increases in fibre tensile properties (presumably due to fibre reinforcement by hydrolysed chitosan fragments penetrating the alginate structure). However, when the chitosan content was over 25% w/w (from treatment using 5% w/w hydrolysed chitosan) the resultant fibres became too brittle. This pattern was also observed in the case of base A3 alginate fibres. Treatment of base A3 alginate fibres with 2% w/w hydrolysed C3 chitosan produced weak fibres, and treatment with 3% produced fibres with improved tensile properties, whilst treatment with 3.9% w/w hydrolysed C3 chitosan resulted in a reduction in the tensile properties. The results for the A1 base alginate fibres treated with GlcN.HCl and GlcNAc (Table 5) show that very little incorporation into the fibre occurred, indicating that very small fragments are not retained and hence do not strengthen the A1 base

Table 5
Composition and analysis of control fibres

Dope (% w/w)	Draw ratio	1st bath	2nd bath	3rd bath	Moisture (% w/w)	Ash (% w/w)	Chitosan (% w/w)	Elongation (%)	Tenacity (cN/dtex)
A1 (6%)	1.18	HCl (0.2 M)	Water	–	5.4	1.4	0.0	20.4	2.2
A2 (6%)	1.09	HCl (0.5 M)	Water	–	7.8	1.3	0.0	5.8	0.8
A3 (4%)	1.09	HCl (0.2 M)	Water	–	9.4	1.1	0.0	13.1	1.2
A1 (6%)	1.18	HCl (0.2 M)	Water	GlcN.HCl (3.9%)	9.8	0.3	0.0	tw	tw
A1 (6%)	1.18	HCl (0.2 M)	Water	GlcNAc (3.9%)	9.6	0.3	1.8	tw	tw

Table 6
Composition and analysis of unhydrolysed chitosan/alginate fibres

Dope (% w/w)	Draw ratio	1st Bath	2nd Bath	3rd Bath	Moisture (% w/w)	Ash (% w/w)	Chitosan (% w/w)	Elongation (%)	Tenacity (cN/dtex)
A1 (6%)	1.18	HCl (0.2 M)	Water	C1 (3.2%)	12.5	0.7	2.1	23.4	2.0
A2 (6%)	1.09	HCl (0.5 M)	Water	C1 (3.2%)	11.6	0.8	5.5	tb	tb
A3 (4%)	1.09	HCl (0.2 M)	Water	C1 (3.2%)	12.4	1.2	2.8	tb	tb
A1 (6%)	1.18	HCl (0.2 M)	–	C1 (3.2%)	12.3	0.4	1.1	15.0	1.0
A2 (6%)	1.09	HCl (0.5 M)	–	C1 (3.2%)	11.4	0.7	0.9	tb	tb
A3 (4%)	1.09	HCl (0.2 M)	–	C1 (3.2%)	12.1	0.8	1.1	tb	tb
A1 (6%)	1.18	HCl (0.2 M)	Water	C2 (2%)	11.2	1.6	0.7	29.1	1.4
A2 (6%)	1.09	HCl (0.5 M)	Water	C2 (2%)	11.2	1.0	1.7	4.8	0.6
A3 (4%)	1.09	HCl (0.2 M)	Water	C2 (2%)	11.4	1.1	2.3	10.5	0.7
A1 (6%)	1.18	HCl (0.2 M)	–	C2 (2%)	11.6	1.0	1.1	12.5	1.0
A2 (6%)	1.09	HCl (0.5 M)	–	C2 (2%)	11.3	0.8	2.3	10.9	1.1
A3 (4%)	1.09	HCl (0.2 M)	–	C2 (2%)	12.3	0.9	0.1	tb	tb

(tb = too brittle to test).

alginate fibre, in fact the treatment process resulted in significant weakening of the A1 base alginate fibre.

3.4. Microscopic analysis

Selected fibres were examined by optical microscopy and the resultant images analysed using Image Pro software in order to compare and contrast dimensions (Fig. 7). Untreated base A1 alginate fibres (Fig. 7(i)) were ~7.7–14.8 μm wide (mean 10.0 ± 2.2 μm), base A1 alginate fibres treated with 2% w/w hydrolysed C3 chitosan (Fig. 7(ii)) were ~7.9–18.4 μm wide (mean 13.2 ± 3.2 μm), and base A1 alginate fibres treated with 5% w/w hydrolysed C3 chitosan (Fig. 7(iii)) were ~7.0–13.8 μm wide (mean 9.9 ± 1.8 μm). Despite circular holes in the spinneret, extruded fibres appeared partially flat/ribbon like, were relatively uniform and displayed some striation along the fibre length.

3.5. Fibre staining

Incubation with Shirlastain A (1% w/w) had no visual effect on untreated A1 alginate fibres (i.e. colour remained off-white). However, A1 alginate fibres treated with hydrolysed C3 chitosan (2, 3.9 and 5% w/w) underwent different levels of staining, from light pink to increasingly pinkish-red. The specific location of chitosan in these fibres (i.e. penetration or surface coating) requires investigation, along with the staining of pure chitosan fibres, in order to interpret these results. A higher concentration of chitosan at the fibre surface may result in the darker staining effects observed at higher chitosan concentrations.

Table 7
Composition and analysis of hydrolysed chitosan/alginate fibres

Dope (% w/w)	Draw ratio	1st Bath	3rd Bath	Moisture (% w/w)	Ash (% w/w)	Chitosan (% w/w)	Elongation (%)	Tenacity (cN/dtex)
A1 (6%)	1.18	HCl (0.2 M)	Hydrol. C1 (3.2%)	11.9	0.3	7.4	20.5	2.5
A2 (6%)	1.18	HCl (0.5 M)	Hydrol. C1 (3.2%)	11.8	0.3	9.6	5.6	1.2
A3 (4%)	1.09	HCl (0.2 M)	Hydrol. C1 (3.2%)	11.8	0.4	8.8	7.3	0.8
A1 (6%)	1.18	HCl (0.2 M)	Hydrol. C2 (2%)	11.7	0.1	11.9	29.3	2.7
A2 (6%)	1.18	HCl (0.5 M)	Hydrol. C2 (2%)	11.7	0.3	16.3	10.9	1.8
A3 (4%)	1.09	HCl (0.2 M)	Hydrol. C2 (2%)	11.4	0.4	10.1	6.0	0.7
A1 (6%)	1.18	HCl (0.2 M)	Hydrol. C3 (2%)	9.9	0.7	5.4	15.5	1.5
A1 (6%)	1.18	HCl (0.2 M)	Hydrol. C3 (3%)	10.7	0.4	14.7	19.2	1.5
A1 (6%)	1.18	HCl (0.2 M)	Hydrol. C3 (3.9%)	10.4	0.9	21.2	23.3	1.7
A1 (6%)	1.18	HCl (0.2 M)	Hydrol. C3 (5.0%)	10.1	1.0	25.2	tb	tb
A3 (4%)	1.18	HCl (0.5 M)	Hydrol. C3 (2%)	11.4	0.6	15.5	tw	tw
A3 (4%)	1.18	HCl (0.5 M)	Hydrol. C3 (3%)	11.7	0.4	23.3	18.5	2.4
A3 (4%)	1.18	HCl (0.5 M)	Hydrol. C3 (3.9%)	11.9	0.1	22.0	10.1	1.4

(2nd bath contained water in all cases; tw = too weak; tb = too brittle).

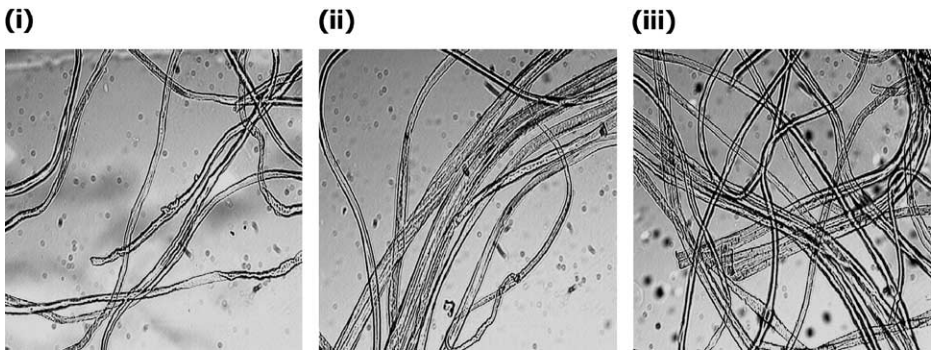

Fig. 7. Optical micrographs (× 400) of A1 alginate fibres treated with hydrolysed C3 chitosan: (i) 0% w/w (i.e. untreated); (ii) 2% w/w; (iii) 5% w/w.

3.6. Antibacterial testing

The antibacterial properties of selected fibres were investigated (Table 8) since one of the aims of significant chitosan incorporation into alginate fibre is to impart antibacterial activity to the fibre. The results presented in Table 8 show that A1 alginate fibres treated with hydrolysed C3 chitosan (3% w/w) give the most timely and effective bacterial reduction with initial use (i.e. likely when first applied to a wound). However, A1 alginate fibres treated with hydrolysed C3 chitosan (1% w/w) are nearly as effective initially as the 3%, and are more effective at leaching antibacterial components, presumably hydrolysed chitosan fragments (test condition (ii). All of the re-used fibres are still capable of imparting good antibacterial properties (test condition (iii).

Table 8
Antibacterial testing of hydrolysed chitosan/alginate fibres

Sample/incubation time (hours)	% Reduction		
	(i) Original fibres	(ii) Re-used solution after fibre removal	(iii) Re-used fibres
A1 + hydrolysed C3 (0%):			
1	95.87	>99.90	99.30
3	>99.86	>99.90	>99.97
6	>99.99	>99.90	>99.97
24	>99.99	>99.90	>99.97
A1 + hydrolysed C3 (1%):			
1	99.61	>99.92	99.52
3	>99.99	>99.92	>99.97
6	>99.99	>99.92	>99.97
24	>99.99	>99.92	>99.97
A1 + hydrolysed C3 (2%):			
1	99.91	>99.87	98.57
3	>99.99	>99.87	>99.97
6	>99.99	>99.87	>99.97
24	>99.99	>99.87	>99.97
A1 + hydrolysed C3 (3%):			
1	99.98	>99.84	99.09
3	>99.99	>99.84	>99.97
6	>99.99	>99.84	>99.97
24	>99.99	>99.84	>99.97
A1 + hydrolysed C3 (5%):			
1	92.33	91.39	96.60
3	>99.99	>99.98	99.94
6	>99.99	>99.98	>99.97
24	>99.99	>99.98	>99.97
A1 + dialysed unhydrolysed C3 (3.9%)			
1	78.08	–	–
3	86.76	–	–
6	89.37	–	–
24	99.99	–	–
A1 + dialysed hydrolysed C3 (3.9%)			
1	88.48	–	–
3	98.32	–	–
6	99.47	–	–
24	99.99	–	–

The slower antibacterial properties associated with A1 alginate fibres treated with the 0% chitosan bath (i.e. hydrolysis agents only), compared to fibres treated with hydrolysed chitosan, will be due to the acidity/antibacterial properties of the A1 alginate fibres only. In comparison, chitosan and hydrolysed chitosan solutions that have been dialysed to neutrality clearly show the time release antibacterial effect of the chitosan only. The antibacterial effect of dialysed hydrolysed chitosan is more rapid than unhydrolysed chitosan, presumably because the bacteria must first break down the latter material.

The antibacterial effects of chitosans and chitosan oligomers have been investigated previously (Martorana, Gentili, Marzocca, & Rodriguez, 2002; No, Park, Lee and Meyers, 2002), and have been shown to be dependent upon chitosan molecular size and bacterium type.

4. Conclusions

Achieved levels of unhydrolysed chitosan incorporation (~0–6% w/w) into alginate fibres were significantly higher than prior art. The fact that such treatment had no reinforcing effects, i.e. fibre strength did not increase, implied that the chitosan was more like a coating and not penetrating the alginate fibre.

A range of chitosan hydrolysates were prepared and analysed, and were used to produce hydrolysed chitosan/alginate fibres, resulting in significant increases in levels of chitosan incorporation (~7–25% w/w). Lowering of

the molecular weight of the chitosan clearly had a positive effect on its ability to penetrate the alginate fibres, not only increasing fibre chitosan content, but also reinforcing fibre structure and thus enhancing tensile properties (compared with unhydrolysed chitosan/alginate fibres). However, too high fibre hydrolysed chitosan contents resulted in brittleness/weakness of the fibres.

Antibacterial testing of hydrolysed chitosan/alginate fibres demonstrated their antibacterial effect (in terms of bacterial reduction) with initial use, and their ability to provide a slow release/leaching of antibacterially active components (presumably hydrolysed chitosan fragments).

The overall aims of these investigations were to maximise chitosan content (and thus provide sufficient antibacterial activity) whilst retaining the necessary physical properties (i.e. satisfactory textile processing ability), since these are important factors with respect to potential application in wound dressings.

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