Chitosan Film Acylation and Effects on Biodegradability

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ABSTRACT: Chitosan films were acylated under heterogeneous conditions in methanol with acetic and hexanoic anhydrides and characterized by proton nuclear magnetic resonance, elemental analysis, and multiple internal reflective Fourier transform infrared spectroscopy. The disappearance of the NH_2 vibrational band at 1590 cm^{-1}, the appearance of the amide II band at 1555 cm^{-1}, and the relatively low intensity of the ester band at 1735 cm^{-1} showed that acylation at the surface was site-selective for the amino (N) functionalities. Furthermore, N-acylation at the surface region appeared complete within 1 h. The acylated chitosan films were fractionated in aqueous acetic acid for compositional analysis. Acetylation of chitosan films for 3 h gave 52% of aqueous acetic acid insoluble chitin (outer film region) and 48% unreacted chitosan. In contrast, 3 h hexanoylation reactions resulted in <1% hexanoylation and a >99% aqueous acetic acid soluble product. Thus, film N-acetylation was more rapid than N-hexanoylation. Moreover, acetylation resulted in the formation of discrete outer chitin layers and an unreacted chitosan interior. The thickness of these film regions with different compositions may be controlled by the reaction conditions. Biodegradation studies of the acylated chitosan films carried out in laboratory-scale aerobic thermophilic compost reactors revealed that the formation of chitin at the film surface enhanced the biodegradability of the films. Specifically, the 3 h acetylated chitosan film (0.045 mm thickness) showed 100% weight loss during a 28 day exposure, whereas unmodified chitosan showed no significant weight loss after 35 days.

Introduction

Chitin, the major polysaccharide of the exoskeletons of insects and shells of crustaceans, has $\beta(1-4)$-linked *N*-acetylglucosamine repeat units and is the second most abundant form of polymerized carbon found in nature.[1] Chitosan, the fully or partially deacetylated form of chitin, contains 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNac, A) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN, D) residues. Chitosan is found in the cell walls of some fungi such as *Mucor rouxii*.[2−4] Chitin and chitosan have been shown useful as chelating agents,[5−7] drug carriers,[8] membranes,[9,10] water treatment additives,[11] biodegradable pressure-sensitive adhesive tape,[12] and wound-healing agents and for a number of other important applications.[13−16]

The structure of chitosan is useful to the synthetic chemist interested in site-selective modification due to the distinctly different reactivities of the amino group at the C2 position and the primary and secondary hydroxyl groups at C6 and C3 positions. It is therefore not surprising that a number of investigators have reported work where site-selective *N*-acylchitosan derivatives were prepared.[7,17−20]

We have previously studied the biodegradability of chitosan film (15% N-acetylated) using laboratory-scale marine- and soil-simulated tests.[21] On the basis of the weight loss, it was found that chitosan film degraded more rapidly than cellophane and poly[(3-hydroxybutyrate)-*co*-(3-hydroxyvalerate)] films in both exposure environments. Laham *et al.* reported that for polyethylene−chitin and polyethylene−chitosan blends containing 90% polyethylene, substantial weight loss of the chitosan (73%) and chitin (85%) components was found after 6 month incubations in soil environments.[22] Also, it has been demonstrated that chitosan is degraded by chitosanases produced by microbial genera such as *Arthrobacter*, *Bacillus*, *Streptomyces*, *Aspergillus*, and *Penicillium* which are found in a wide range of ecosystems.[23] An interesting study by Varum *et al.* concerning the specificity of a chitosan-degrading enzyme extracted from the stomach contents of Atlantic salmon showed that it did not degrade deacetylated (0% DA) chitosan but showed substantial activity for the degradation of 15% N-acetylated chitosan.[24] This illustrates how specific degrees of acetylation may be required by some chitosan degrading enzyme systems.

Chitosan is readily processible into fibers and films from aqueous acid solution.[25] Bulk films and fibers have been modified by cross-linking with epichlorohydrin.[26,27] The modification of solution-processed chitosan film at the surface and beyond to tailor film physical and biological properties would be useful. In this study, the heterogeneous acetylation and hexanoylation of chitosan films using acetic and hexanoic anhydrides, respectively, were carried out. The degrees of acetylation and hexanoylation at the film surface and film interior were analyzed by multiple internal reflective Fourier transform infrared (MIR-FTIR) spectroscopy, proton nuclear magnetic resonance (^{1}H NMR), and elemental analyses (EA). Also, MIR-FTIR gave information on the degree of site selectivity achieved during film modification. Studies carried out to determine the relationship between film acylation and biodegradability in simulated in-laboratory compost exposures showed important effects that will be useful in designing chitosan film surfaces to have either enhanced or depressed degradability.

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Experimental Section

Film Preparation. Chitosan (15 g; Protan Laboratories Inc.) was dissolved with stirring in distilled water (1 L) containing 2% (v/v) acetic acid. Insoluble substances were removed by filtration through a medium-pore-size glass funnel to yield a chitosan solution (*ca.* 1.5%, w/v). Portions (75 mL) of this solution were cast into films using a 17.8 × 17.8 cm plastic mold. After solvent evaporation, the films were soaked in 1 N NaOH for 1 h to remove residue acetic acid. The films were then washed with a large quantity of distilled water several times until the wash was neutral followed by drying first on a piece of glass with the film's edges clamped and, finally, in a vacuum oven (50 °C, 0.5 mmHg, 24 h). The resulting films were 0.045 ± 0.003 mm thick.

Modification Reactions. The procedure used was a modification of that previously reported.[28] Chitosan films were reacted with 1.0 M of either acetic or hexanoic anhydride in methanol (acetic and hexanoic anhydrides were purchased from Aldrich with >99% purity and used as received.). The ratio of anhydride to amino functionalities in the film was no less than 25. The reactions were carried out with agitation using an orbital shaker with a shaking rate of *ca.* 120 rpm at 25 °C for various time periods as specified below. Reactions were terminated by removal of the films from the reaction solution and washing the films over a 15 min time period with five 100 mL portions of methanol. The films were then dried (50 °C, 0.5 mmHg, 12 h) prior to characterization.

Fractionation of Modified Chitosan Films. The modified chitosan films (~1.1 g) were placed in 2% (v/v) acetic acid aqueous solutions (75 mL) with stirring for 24 h; the solubilized polymer was separated from insoluble material by filtration through a medium-pore-size glass funnel. The insoluble fractions (IS) were washed two times with 10 mL portions of 2% aqueous acetic acid solution, placed in a 1 N NaOH solution for 1 h, and then washed with large volumes of distilled water several times until the distilled water wash was neutral. The soluble fractions (S) were cast into films as described earlier.

Product Characterization. ^{1}H NMR were recorded on a Brucker WP-270 SY spectrometer at 270 MHz using sodium 3-(trimethylsilyl)propionate as an internal reference at 0.00 ppm and D_2O with 2% (v/v) acetic acid-d_4 as the solvent. The experimental parameters were as follows: polymer concentration, 1%, w/w; temperature, 70 °C; pulse width, 3.0 μs; 32 data points; relaxation delay, 2.0 s; and 200−300 transients. Quantitative measurement of peak areas was made by cutting and weighing. The degrees of acetylation and hexanoylation (reported in units of percent) are defined as the average number of acetyl and hexanoyl groups, respectively, per repeat unit multiplied by 100. The degree of acetylation (DA) was calculated using the peak areas (PA) of H1(D), H1(A), and -CH_3 of *N*-acetyl substituents (see Figure 2) as follows: $DA = PA_{CH_3}/3(PA_{H1(D)} + PA_{H1(A)}) \times 100$ (see results and disscussion section for peak assignments). The degree of hexanoylation (DH) was estimated similarly but using the ^{1}H NMR signal at 0.90 ppm due to the methyl group of hexanoyl substituents. MIR-FTIR spectra were recorded on a Mattson Instruments Galaxy Series FTIR 2020 spectrometer with 128 scans. A crystal of KRS-5 (45 deg. para.; Spectra-Tech, Inc., Stamford, CT) was used as the reflection prism. Samples were vigorously dried (100 °C, 50 μmHg, 24 h) prior to submission of samples for elemental analyses. All elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA. The degree of acetylation determined by EA was calculated using the following equation: $DA = [(C/N - 5.14)/1.72] \times 100\%$, where C/N is the ratio (w/w) of carbon to nitrogen. The degree of hexanoylation was calculated assuming products having DA = 20% using the following equation: $DH = [(C/N - 5.48)/5.14] \times 100\%$.

Biodegradation in Compost Exposure. Biodegradability was determined based on film weight loss measurements. Tests were conducted in laboratory-scale aerobic thermophilic compost reactors at 53 °C, 60% moisture, and using a synthetic municipal waste mixture. Details of the experimental protocol for this method have been described elsewhere.[29] The studies were conducted using three replicate test reactors, three replicate samples in each test reactor for each exposure time, and a poisoned control vessel. Therefore, results on weight loss were based on nine samples. The poisoned vessel served as an abiotic control which was maintained identically with the bioactive test reactors.

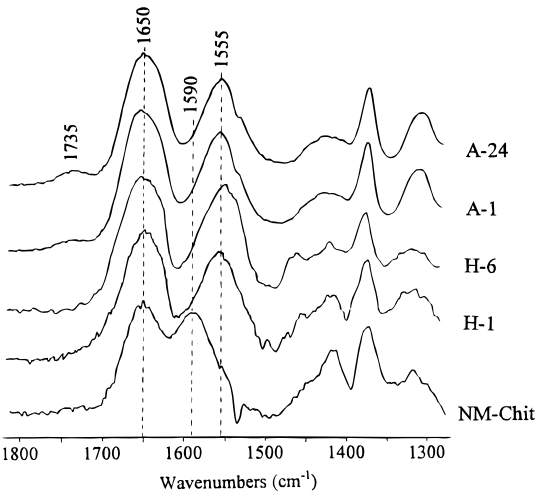
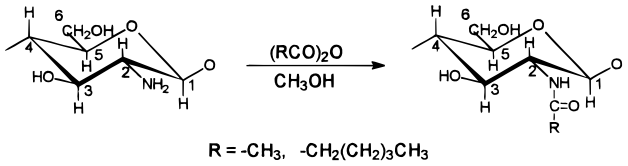


Figure 1. MIR-FTIR spectra of NM-chit, H-1, H-6, A-1, and A-24.

Scheme 1. Heterogeneous Acylation Reaction of Chitosan



Scanning Electronic Microsopy (SEM). Film surfaces were observed with an AMRAY 1400 SEM instrument. Visible debris were carefully removed; the films were dried (50 °C, 0.5 mmHg, 16 h) and then sputter coated with carbon. Samples were mounted onto metal studs. The electron-accelerating voltage and magnifications were 10 kV and 500 times, respectively.

Results and Discussion

MIR-FTIR Studies. The acylation of chitosan can be achieved in homogeneous reaction media by, for example, carrying out reactions of chitosan and anhydrides in acetic acid/methanol solutions.[30] Unfortunately, gelation often occurs during the course of acylation reactions to form products which are difficult to dissolve for subsequent processing into films and fibers.[17] A route which circumvents this difficulty is to first process chitosan using acetic acid aqueous solutions and then carry out modifications of the resulting formed materials by heterogeneous processes. In the present work, chitosan films were acylated with acetic and hexanoic anhydrides in methanol under heterogeneous conditions as shown in Scheme 1, adopting the methodology previously described by Moore *et al.*[28]

The acylated chitosan films were characterized by MIR-FTIR to study the extent of acylation at the film surface. This technique allows visualization of the film surface to a depth of <1 μm.[31] Figure 1 shows the MIR-FTIR spectra of chitosan films which were reacted for 1 and 24 h with acetic anhydride (products A-1 and A-24, respectively) and for 1 and 6 h with hexanoic anhydride (H-1 and H-6) and the not-modified chitosan (NM-chit) (see Table 1 footnote *a* for abbreviations used). The absorption peaks at *ca.* 1650, 1590, and 1555 cm^{-1} were assigned to the carbonyl stretching of secondary amides (amide I band), N−H bending vibrations of

Table 1. Compositional Analysis of Nonmodified and Acylated Chitosan Films

product and fraction[a]	DA (%) by EA[b]	DA (%) by NMR[c]	DH (%) by EA[d]	DH (%) by NMR[e]	wt (%)
NM-chit	20	14 ± 2			
A-1	39				100
A-1-S	25	17 ± 2			79
A-1-IS	97				21
A-3	63				100
A-3-S	23	17 ± 2			48
A-3-IS	107				52
A-24-IS	110				100
H-1-S		14 ± 2	<0.5	<1	>99
H-3-S		16 ± 2	<0.5	<1	>99
H-5.25-S		18 ± 2	<0.5	<1	92
H-5.25-IS			66		8

[a] 2% aqueous acetic acid solution was used as the solvent for the fractionation. NM-chit denotes not-modified chitosan. The abbreviations for remaining samples are as follows: acetylated (A) and hexanoylated (H); reaction times 1, 3, 5.25, and 24 h; and product fraction soluble (S) and insoluble (IS), respectively, in 2% aqueous acetic acid. If there is no designation for S or IS, then the sample analyzed was unfractionated. [b] Degree of acetylation (DA) determined by elemental analysis, EA (see Experimental Section). [c] DA determined by ^{1}H NMR (see Experimental Section). [d] Degree of hexanoylation (DH) determined by EA (see Experimental Section). [e] DH determined by ^{1}H NMR (see Experimental Section).

nonacylated 2-aminoglucose primary amines, and N−H bending vibrations of secondary amides (amide II band), respectively.[13,17,32,33] The NM-chit starting material in this work was reported by the manufacturer to have a DA of about 15%. For this sample, the presence of both 2-aminoglucose and 2-acetamidoglucose chitosan repeat units are indicated by bands at 1650, 1590, and 1555 cm^{-1}. After a reaction time of 1 h for both acetylation and hexanoylation, the vibrational band corresponding to unreacted primary amino groups at 1590 cm^{-1} is not apparent while prominant bands at 1650 and 1555 cm^{-1} are observed. Furthermore, a relatively weak vibrational band at *ca.* 1735 cm^{-1} (due to O-acylation) is noticeable after a 1 h acetylation reaction which shows only a small increase in intensity even after an extended reaction time of 24 h. Hexanoylation reactions show no observable absorbance at 1735 cm^{-1} even after a 6 h reaction period. These results indicate that, in the region defined as the surface based on MIR-FTIR measurements, N-acylation occurs at much greater rates and to greater extents than O-acylation. Thus, site-selectively modified chitosan derivatives were obtained. The observation that low levels of O-acylation occurs during acetylation but not hexanoylation reactions is evidence that acetylation of chitosan films occurs to greater extents than film hexanoylation.

Solubility and Fractionation of Modified Chitosan Films. Acetylated and hexanoylated chitosan films dissolved in 2% aqueous acetic acid solutions to variable extents depending on the acylation reagent and the reaction time. While NM-chit film dissolves readily in 2% aqueous acetic acid, reactions of the chitosan film for 24 h with acetic anhydride renders the films insoluble in this solvent. This is evidence that these films were extensively N-acetyated throughout the bulk. In agreement with the above, it has been reported that chitosan with DA's of <18%, 45%, and >60% in 5% aqueous acetic acid are soluble, swelled, and insoluble, respectively.[7,17] The differences in solubility of chitosan in 2% aqueous acetic acid as a function of substitution provided a convenient route for product fractionation (Table 1). As expected, the % IS material increased with

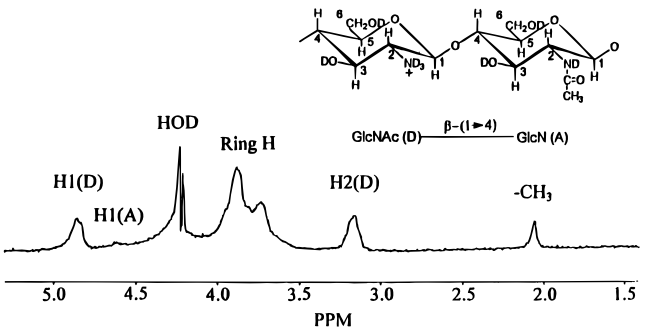


Figure 2. ^{1}H NMR spectrum recorded at 270 MHz of NM-chit in D_2O with 2% (v/v) acetic acid-d_4.

reaction time for the acetylated films; IS fractions of acetylated films with reaction times of 1, 3, and 24 h are 21%, 52%, and 100%, respectively. In contrast, acylation reactions carried out using hexanoic anhydride appeared to proceed at a slower rate and to a lower extent than acetylation reactions. Attempts to fractionate 1 and 3 h hexanoylated chitosan films were not successful since the films had negligible IS fraction weight percentages. After a 5.25 h hexanoylation reaction, the IS fraction was only 8%. The apparent faster reactivity of chitosan films with acetic anhydride relative to hexanoic anhydride based on solubility results was further substantiated by compositional analysis of S and IS fractions.

^{1}H NMR and Elemental Analysis. The DA and DH of the unfractionated, S, and IS samples were determined by elemental analysis. ^{1}H NMR was also used to determine the DA and DH of S product fractions. In Figure 2, a representitive ^{1}H-NMR spectrum of NM-chit is shown along with peak assignments for D and A repeat units. Peak assignments were based on previously published work,[34,35] and the calculation of DA and DH from spectral integration of proton signals is described in the Experimental Section. The results of DA and DH analyses for unfractionated, S, and IS fractions are given in Table 1. ^{1}H NMR studies indicated that NM-chit film had a DA of 14 ± 2%, in good agreement with that given by the supplier (15%) and from elemental analysis which indicated a DA value of 20%. Interestingly, A-1-S and A-3-S product fractions had DA values that closely approximated that of NM-chit (see Table 1). DH for H-5.25-S was low (<1%), such that weak ^{1}H NMR signal resonances assignable to hexanoyl substituents were barely distinguishable from base-line noise (see Table 1 and Experimental Section). Furthermore, the DA values of the A-1-IS, A-3-IS, and A-24-IS product fractions were significantly higher (97%, 107%, and 110%, respectively) having *ca.* 1 acetyl substituent/residue. On the basis of the MIR-FTIR analysis of the surface region which is part of the IS fractions, it can be concluded that acetylation occurred site-specifically at primary amino functionalities. From the above, we conclude that chitosan films acetylated by the procedure described in this paper are produced with an inner core that remains unreacted and an outer core that approximates completely N-acetylated chitosan or chitin. Moreover, the results suggest that the thickness of the outer film chitin region can be carefully selected by the reaction conditions and is ultimately determined by the rate of anhydride diffusion into the chitin−chitosan interfacial region.

The modification of chitosan films with hexanoic anhydride is considerably slower than that observed with acetic anhydride (see discussion above). Figure 1

shows that the film surface region as defined by the MIR-FTIR experiment is N-hexanoylated to high extents after only a 1 h reaction time. However, after a 5.25 h hexanoylation, the IS film fraction (H-5.25-IS) possessed only 8% by weight and exhibited a DH of 66%, *ca.* 14% below that for complete N-acylation. Hexanoylation reactions carried out for 1 and 3 h (H-1 and H-3, respectively) resulted in films that showed negligible IS fractions and S fractions of low (<1%) DH. The observed comparative reactivity of acetic and hexanoic anhydrides in methanol with chitosan film is in contrast to that previously reported by Moore *et al.*[28] Under similar reaction conditions as we have used, Moore *et al.* reported that the two anhydrides required approximately similar reaction times for complete N-acylation and that the Arrhenius energy of activation was relatively smaller for the latter reaction. In addition, these workers did not recognize the high selectivity for N-acylation achieved using this method for film acylation and utilized, therefore, a de-O-acetylation step subsequent to reactions with anhydrides to prepare N-acylated chitosans. Our results can be rationalized by considering that the rate of diffusion of hexanoic anhydride through an outer N-hexanoylated film layer to the inner chitosan core is slow relative to acetic anhydride diffusion through a chitin outer layer. Furthermore, differences in reaction rates between the two anhydrides and chitosan film are expected where the larger hexanoic anhydride might have greater difficulty in achieving the required geometry for effective molecular collisions. The dynamic changes in outer layer thickness and nature of the boundary region between inner chitosan and outer acylated film regions, as well as film crystalline morphology, serve as challenging problems that require consideration in further understanding the observed film modifications.

Biodegradation of Chitosan and N-Acylated Chitosan Films. As discussed earlier, chitosan as well as cross-linked chitosan appeared biodegradable when exposed to mesophilic marine and soil environments. In the work reported herein, the degradability of NM-chit and partially N-acylated chitosan films was assessed by exposure of the films to laboratory-scale aerobic thermophilic compost reactors and abiotic control vessels. The normalized weight loss of the films was determined by measuring the weight loss and dividing by the initial surface area (units of μg/mm^2). The results obtained are presented in Figure 3. The rationale for this approach to data manipulation which takes into account effects of the substrate surface area on the weight loss rate has been described elsewhere.[36] The weight loss observed for films in the control or abiotic vessels was little to none. The incubation of NM-chit in bioactive test vessels resulted in no weight loss after exposure periods of up to 35 days. However, after *ca.* 21 days, the NM-chit films which were initially transparent, faint yellowish in color, and flexible turned brown and became brittle. These color changes and embrittlement indicate that NM-chit has undergone primary stages of environmental degradation. In contrast to the NM-chit samples, chitosan films reacted with acetic anhydride for 1 (A-1) and 3 (A-3) h showed dramatically enhanced normalized weight loss. In fact, after a 28 day exposure time, the A-3 film had completely disappeared based on visual inspection of the residual test waste matrix. Moreover, a comparison of films A-1 and A-3 showed that the latter, which presumably has a thicker chitin outer layer, was more readily degraded. When one considers

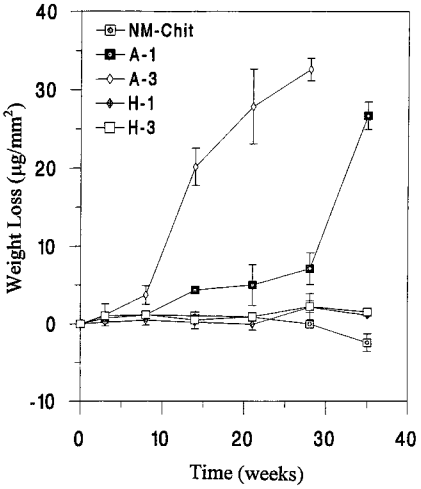


Figure 3. Normalized weight loss as a function of the incubation time for NM-chit and modified chitosan films A-1, A-3, H-1, and H-3 exposed in laboratory-scale aerobic thermophilic compost reactors.

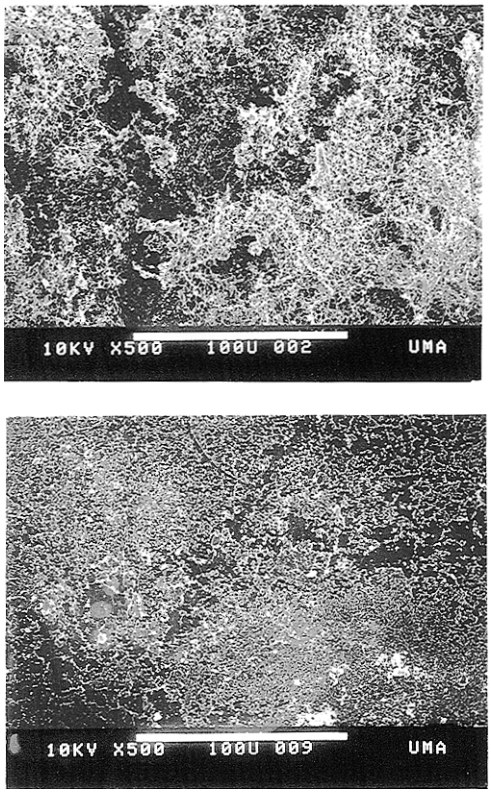


Figure 4. Scanning electron micrographs of the degraded samples by aerobic thermophilic compost: A-3 after 14 days (top) and NM-chit after 21 days (bottom). Scale bar indicates 100 μm (reproduced at 56% of original size).

that chitosan is rare in nature whereas chitin is highly abundant, it is reasonable to observe an acceleration in the biodegradation rate of chitosan films upon transformation of the film surface to chitin. SEM photographs were obtained for films A-3 and NM-chit after 14 and 21 day exposure times, respectively (see Figure 4). The exposed A-3 sample (Figure 4, top) showed bigger and deeper holes/pits which were more random in distribution relative to the NM-chit sample (Figure 4, bottom). The '*honeycombed*' holes observed in the A-3 samples were most likely due to microbial colonization and were not observed for exposed NM-chit.

It is interesting to consider that film A-1 still had appreciable quantities of residual chitosan after 1 h

acetylation (*ca.* 79% by wt). Thus, the degradation of chitosan in the film interior may be initiated by microorganisms that degrade chitin rapidly but are also able to more slowly utilize chitosan. Therefore, it was demonstrated that by the proper alteration of the film surface chemistry, an otherwise apparently biologically inert polymer (based on weight loss measurements) showed significantly enhanced biodegradation rates. That chitosan was previously shown to be readily degradable in aerobic mesophilic marine and soil environments,[21,22] where, in this work, films were exposed to aerobic thermophilic conditions, raises the question as to whether chitosan-degrading organisms are more prevelant under mesophilic conditions.

The biodegradability of chitosan films reacted with hexanoic anhydride for 1 and 3 h (H-1 and H-3, respectively) was also studied under the same exposure conditions. In contrast to accelerated film weight loss due to acetylation, films H-1 and H-3 showed little to no weight loss after 35 day exposure periods. The observed differences between film acetylation and hexanoylation are reasonable when it is considered that chitosan N-acetylation regenerated the natural biological macromolecule chitin, whereas hexanoylated chitosan is not, to our knowledge, found in nature.

Conclusions

Studies utilizing MIR-FTIR indicated that, after 1 h reactions of chitosan films with acetic and hexanoic anhydrides in methanol, site-selective N-acetylation and N-hexanoylation at the film surfaces appeared complete. On the basis of compositional analysis of modified chitosan films, it was found that acetylation beyond the surface proceeded more rapidly and to higher extents than hexanoylation. Furthermore, film acetylation resulted in three distinct layers consisting of an inner core that remained unreacted sandwiched between outer layers of chitin. The thickness of the respective layers was a function of the reaction time. Biodegradation tests showed that nonmodified or hexanoylated chitosan films exhibited no apparent weight loss for 35 day exposures in laboratory-scale aerobic thermophilic test reactors. In contrast, by regenerating chitin at film surfaces via N-acetylation, the rate of film biodegradation was dramatically enhanced.

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