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# Controlling cell adhesion and degradation of chitosan films by *N*-acetylation

Thomas Freier[a,b], Hui Shan Koh[a], Karineh Kazazian[a], Molly S. Shoichet[a,b,c,*]

[a]*Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ont., Canada M5S 3E5*
[b]*Institute of Biomaterials and Biomedical Engineering, 4 Taddle Creek Road, Room 407, Toronto, Ont., Canada M5S 3G9*
[c]*Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ont., Canada M5S 3H6*

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**Abstract**

As part of our ongoing effort to develop a biodegradable nerve guidance channel based on chitin/chitosan, we conducted a systematic in vitro study on the biodegradation and neural cell compatibility of chitosan and *N*-acetylated chitosan. The in vitro degradation (pH 7.4, 37 °C) in the presence of 1.5 μg/ml lysozyme showed a progressive mass loss to greater than 50% within 4 weeks for films with 30–70% acetylation. In contrast, the degradation of samples with very low or high acetylation was minimal over the 4-week period. Neural cell compatibility of chitosan and *N*-acetylated chitosan was tested using primary chick dorsal root ganglion (DRG) neurons. All chitosan-based films showed DRG cell adhesion after 2 days of culture. However, cell viability decreased with increasing acetylation. Chitosan that was 0.5% acetylated had the greatest cell viability, which was approximately 8 times higher than that of chitosan that was 11% acetylated. Chitosan with 0.5% and 11% acetylation showed more and longer neurites than the other samples studied. Thus chitosan amine content can be tuned for optimal biodegradation and cell compatibility, which are important for tissue engineering in the nervous system.


*Keywords:* Chitosan; Chitin; Enzymatic degradation; Nerve regeneration; Tissue engineering

## 1. Introduction

Chitin and chitosan represent a family of biopolymers, made up of β(1→4)-linked *N*-acetyl-D-glucosamine and D-glucosamine subunits. Chitin can be found widely in the exoskeletons of arthropods, shells of crustaceans, and the cuticles of insects [1]. Chitosan, on the other hand, although occurring in some fungi, is produced industrially by alkaline hydrolysis of chitin. Their different solubilities in dilute acids are commonly used to distinguish between the polysaccharides. Chitosan, the soluble form, can have a degree of acetylation (DA) between 0% and about 60%, the upper limit depending on parameters such as processing conditions, molar mass, and solvent characteristics [2].

Chitin and chitosan are widely studied for biomedical applications [3–5]. Their biodegradability and biocompatibility, together with their specific interactions with components of the extracellular matrix and growth factors, have led to their growing use in tissue engineering, such as in the repair of skin, bone, and cartilage [6–8]. Recently, we have introduced a new method for the fabrication of chitin and chitosan tubes for nerve regeneration [9]. The compressive strength of these tubes was found to increase with decreasing acetylation. Both chitin and chitosan support adhesion and differentiation of primary chick dorsal root ganglion (DRG) neurons in vitro, with significantly enhanced neurite outgrowth on chitosan than on chitin films.

*Corresponding author. Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ont., Canada M5S 3E5. Tel.: +1 416 978 1460; fax: +1 416 978 4317.
*E-mail address:* molly@ecf.utoronto.ca (M.S. Shoichet).

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The present study was designed to gain more insight into the influence of the DA on the biodegradation and cell compatibility of chitin and chitosan so that these properties could be tuned for the development of nerve guidance channels. To examine the biodegradation, we used, for the first time, enzyme concentrations similar to those found in the human body and physiological pH in order to simulate in vivo conditions. The DA dependence of the cell compatibility, which has not yet been described for neural cells, was assessed by cell adhesion, viability and differentiation of DRG neurons seeded on the chitosan-based films.

## 2. Materials and methods

### 2.1. Materials

Chitosan, with a molar mass of approximately 400,000 g/mol, was purchased from Fluka (Oakville, Canada) and used as received. The DA was determined to be 10.9% by $^1$H-NMR spectroscopy (see Section 2.3.). Acetic anhydride was purchased from ACP Chemicals (Montreal, Canada). Deuterium oxide ($D_2O$) was purchased from Cambridge Isotope Laboratories (Andover, USA) and deuterium chloride (DCl, 35% in $D_2O$) from CDN Isotopes (Pointe-Claire, Canada). All other chemicals and solvents were purchased from Sigma-Aldrich (Oakville, Canada) and used as received unless otherwise noted. Water was distilled and deionized using Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, USA) at 18 MΩ resistance. Water for cell culture was sterile-filtered through a 0.22 μm Millipore filter (Bedford, USA).

### 2.2. Chitosan and chitin film fabrication

Chitosan with a DA of 0.5% was synthesized by immersing commercially available 10.9% acetylated chitosan flakes in a 40% aqueous solution of sodium hydroxide for 2 h at 110 °C. Chitosan films with DAs of 0.5% and 10.9% were synthesized by pouring a 3% (w/w) solution of chitosan of the desired DA in 2% aqueous acetic acid into Petri dishes, followed by drying at room temperature. The resulting films were treated with a solution of ammonia in methanol/water [10], followed by intense washing with distilled water, and drying.

All other films were prepared by modifying a published procedure [11]. Briefly, a 3% (w/w) solution of chitosan (DA = 10.9%) in 2% aqueous acetic acid was diluted with an equal volume of ethanol and mixed with a 0.1–2.0 molar ratio of acetic anhydride (calculated on the basis of non-acetylated chitosan). The reaction mixtures were poured into Petri dishes and stored at room temperature until dry. The resulting films were treated with a solution of ammonia in methanol/water [10], followed by intense washing with distilled water and drying. Chitin films with 99.2% acetylation were synthesized by immersing 91.5% acetylated chitin films in a 10.0 molar excess of acetic anhydride (calculated on basis of non-acetylated chitosan) in 2% aqueous acetic acid, diluted with an equal volume of ethanol. The films were removed after 24 h, treated with ammonia solution as described above, followed by intense washing with distilled water.

### 2.3. Analysis of the degree of acetylation (DA)

The DA of chitin and chitosan samples was determined using $^1$H-NMR spectroscopy according to a modified published procedure [12,13]. Briefly, samples prepared by addition of more than 0.15 molar excess of acetic anhydride to chitosan were dissolved in a mixture of 25% DCl in $D_2O$. All other samples were dissolved in a mixture of 0.25% DCl in $D_2O$. The concentration of polymer in the solution was approximately 0.5% (w/v) in all cases. The $^1$H-NMR spectra were recorded on a Gemini 300 spectrometer (Varian). The DA was calculated by comparing the integrated area of the signal group of H2–H6 with that of the signal of the methyl group as previously described [12,13].

### 2.4. In vitro degradation

The in vitro degradation of chitosan and chitin films (10 × 5 mm) was followed in 1 ml phosphate-buffered solution (PBS, pH 7.4) at 37 °C containing 1.5 μg/ml lysozyme (hen egg-white, Sigma-Aldrich, Oakville, Canada). The concentration of lysozyme was chosen to correspond to the concentration in human serum [14,15]. Briefly, films of known dry weights were sterilized by autoclaving (120 °C, 20 min) and incubated in the lysozyme solution with gentle mechanical agitation for the period of study. The lysozyme solution was refreshed daily to ensure continuous enzyme activity [16]. After 7, 14, 21 and 28 days, samples were removed from the medium, rinsed with distilled water, dried under vacuum and weighed. The extent of in vitro degradation was expressed as percentage of weight loss of the dried films ($n = 3$ samples of each DA) after lysozyme treatment. To separate between enzymatic degradation and dissolution, control samples were stored for 28 days under the same conditions as described above, but without the addition of lysozyme.

### 2.5. Dorsal root ganglion (DRG) cell culture

Films prepared as described in Section 2.2 were sterilized by autoclaving (120 °C, 20 min) and immersed in Eagle's minimal essential medium (Gibco, Burlington, Canada) for 24 h prior to plating of the DRG neurons.

Lumbar DRG were dissected from E9 White Rock chicks (Brampton, Canada) according to an established protocol [17]. DRG neurons were plated at a density of $1 \times 10^4$ cells/cm$^2$ to facilitate quantitative analysis and cultured on the sample films in a cell culture medium consisting of Eagle's minimal essential medium, 10% horse serum, 0.2 mM glutamine, 1% penicillin/streptomycin, and 50 ng/ml nerve growth factor (NGF, all supplied by Gibco, Burlington, Canada), and incubated (37 °C, 5% $CO_2$ and 100% humidity). After 48 h, 10 random, separate fields per well were recorded at 20× magnification using a phase contrast microscope (LM410, Zeiss) to calculate the average number of adherent cells, the number of cells bearing neurites longer than the cell body length, and the total neurite length, where only those neurites longer than one cell body length were used in this calculation. Each experiment was conducted in triplicate. Results are expressed as mean ± standard deviation. Analysis of variance using an ANOVA Single Factor test was used for the statistical analysis, with 95% confidence.

### 2.6. Cell viability testing

The viability of primary chick DRG neurons cultivated for 2 days on the chitin/chitosan films, as described in Section 2.5, was determined using the MTS assay (Promega, Madison, USA). After 4 h of MTS incubation with the cells, the light absorbance at 490 nm was measured by an ELISA 96-well plate reader (Molecular Devices) and subtracted from that of the controls (without cells) to yield the corrected absorbance. Five samples of each DA were studied.

## 3. Results and discussion

### 3.1. Chitosan and chitin film fabrication

Chitosan and chitin samples were prepared by selective *N*-acetylation of chitosan using acetic anhydride. Solutions of 3% chitosan in 2% aqueous acetic acid were mixed with an equal volume of ethanol, followed by the addition of acetic anhydride. The DA of the reaction product depended on the molar amount of acetic anhydride added per glucosamine subunit of chitosan (Table 1). Films made by addition of 0.3–0.5 molar amount of acetic anhydride showed poor mechanical stability, swelling and partial to complete dissolution when stored in PBS. Addition of more than 0.6 molar amount of acetic anhydride to the chitosan solution led to the formation of chitin gels. These gels have a honeycomb-like morphology which collapses irreversibly during drying and chitin film preparation [18]. The DA of the chitin films plateaued at about 92%, even with the addition of a large excess of acetic anhydride to the chitosan solution. However, by treating a chitin film with a DA of 91.5% with acetic anhydride, we were able to increase its DA to 99.2%. A 10 molar excess of acetic anhydride (based on non-acetylated chitosan) was found to be optimal for this further treatment.

Table 1
Degree of acetylation (DA) of chitosan and *N*-acetylated chitosan samples studied

| Material | DA (%) |
|---|---|
| Hydrolyzed | 0.5 ± 0.0 |
| $R = 0$ | 10.9 ± 0.6 |
| $R = 0.1$ | 20.7 ± 0.5 |
| $R = 0.15$ | 26.3 ± 0.5 |
| $R = 0.2$ | 31.7 ± 0.5 |
| $R = 0.25$ | 37.5 ± 0.4 |
| $R = 0.3$ | 41.1 ± 0.7 |
| $R = 0.35$ | 46.1 ± 1.7 |
| $R = 0.4$ | 51.3 ± 1.2 |
| $R = 0.45$ | 56.2 ± 1.0 |
| $R = 0.5$ | 59.3 ± 0.6 |
| $R = 0.6$ | 66.4 ± 1.3 |
| $R = 1.0$ | 79.5 ± 1.1 |
| $R = 2.0$ | 91.5 ± 0.5 |
| $R = 2.0 + 10.0$ | 99.2 ± 0.5 |

$R$ = molar amount of acetic anhydride used for *N*-acetylation of chitosan, based on the glucosamine subunit of non-acetylated chitosan; for details on sample preparation see Section 2.2.

The selective *N*-acetylation of chitosan using acetic anhydride [11] is a convenient method for the synthesis of chitosan and chitin samples with different DAs. Another simple way to adjust the DA is the deacetylation of chitosan and chitin by alkaline hydrolysis [19]. Both methods allow for the preservation of the natural chemical composition of the polysaccharide, and hence its inherent biological properties. The underlying chemistry is simple and no toxic by-products are formed during the reactions. The ability to vary the DA is a powerful tool for adjusting the properties of chitosan and chitin, including mechanical strength [19,20], biodegradation [20,21], and biocompatibility [10,22–24]. Recently, we described a method for the fabrication of chitin and chitosan nerve guidance channels using *N*-acetylation and *N*-deacetylation reactions. The compressive strengths of these tubes were found to increase greatly with decreasing DA [9]. Herein, we wanted to better understand the influence of the DA on the biodegradation and cell compatibility of chitin and chitosan as these parameters will influence our choice for nerve guidance channel material.

### 3.2. In vitro degradation testing

The results of our biodegradation testing are summarized in Figs. 1 and 2. An accelerated mass loss was

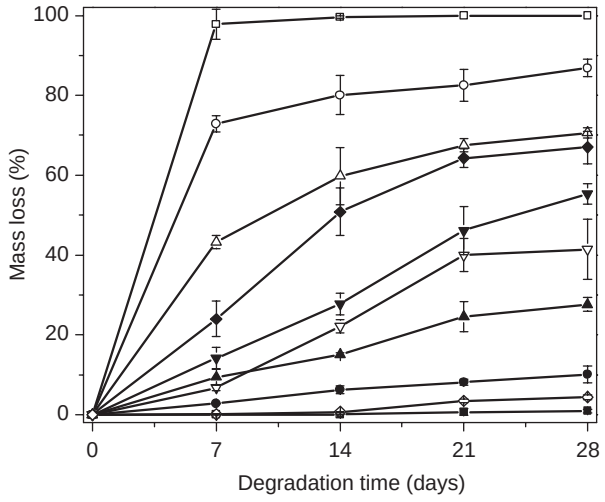


Fig. 1. Progressive mass loss of chitosan and *N*-acetylated chitosan films after storage in PBS at 37 °C with 1.5 μg/ml lysozyme: DA = 0.5% (■); 20.7% (●); 26.3% (▲); 31.7% (▼); 37.5% (◆); 46.1% (□); 59.3% (○); 66.4% (△); 79.5% (▽); 99.2% (◇). Data are shown as mean ± standard deviation ($n = 3$).

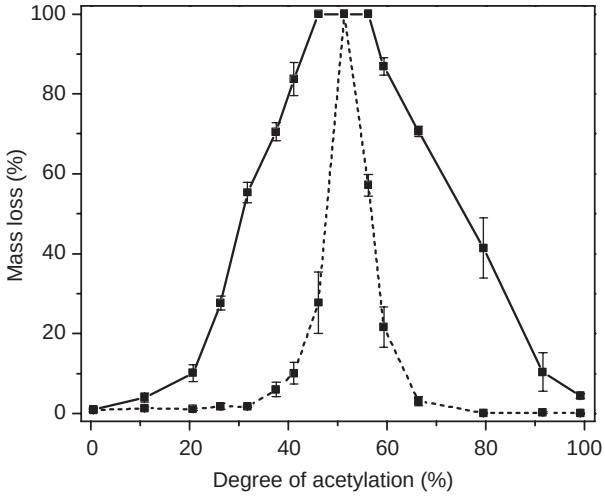


Fig. 2. Mass loss of chitosan and *N*-acetylated chitosan films vs. DA after 28 days in PBS at 37 °C with 1.5 μg/ml lysozyme (solid line) and without lysozyme (dotted line). Data are shown as mean ± standard deviation ($n = 3$).

observed for films having intermediate DAs. For example, samples with a DA of 46.1% showed almost complete degradation within 1 week in lysozyme solution. Other samples with DAs between 30% and 70% showed progressive mass losses up to more than 50% within 4 weeks of degradation (Fig. 1). In contrast, the degradation of samples with very low or high DAs was minimal over the period studied. Nevertheless, chitin (DA = 99.2%) showed a 4 times higher mass loss than chitosan (DA = 0.5%) after 4 weeks of lysozyme treatment.

To distinguish between enzymatic degradation and simple dissolution, we compared the mass loss after 28 days of samples that had been stored in PBS with lysozyme to those that had been stored in PBS without lysozyme (Fig. 2). The aqueous solubility of samples with low or high DAs was negligible and the enzymatic effect clearly visible. Except for chitosan with a DA of 0.5%, all samples showed significant enzymatic degradation. The difference between the mass loss with lysozyme treatment to that without lysozyme treatment, indicating enzymatic degradation, was highest for samples having intermediate DAs (excluding chitosan with a DA of 51.3%, which dissolved completely after 28 days both with and without enzyme addition, thus preventing a distinction between degradation and dissolution to be made).

The degradation of chitosan and chitin has already been tested in a number of in vitro studies [20,21,23,25–31]. However, most of the studies were performed under accelerated conditions using low pH for optimum lysozyme activity as well as high enzyme concentrations. To our knowledge, long-term in vitro studies using physiological pH and enzyme concentrations (as described herein) have not yet been published.

It is well known that, in human serum, *N*-acetylated chitosan is mainly depolymerized enzymatically by lysozyme, and not by other enzymes or other depolymerization mechanisms [21]. The enzyme biodegrades the polysaccharide by hydrolyzing the glycosidic bonds present in the chemical structure. Lysozyme contains a hexameric binding site [25], and hexasaccharide sequences containing 3–4 or more acetylated units contribute mainly to the initial degradation rate of *N*-acetylated chitosan [30]. The pattern of degradation of chitosan and *N*-acetylated chitosan found in our studies can, in part, be explained by this mechanism of enzymatic degradation. After a high initial mass loss, the degradation rate of the samples generally appeared to slow down (Fig. 1), which is a result of the loss of appropriate hexasaccharide sequences with progressing degradation. The lack of consecutive *N*-acetylglucosamine residues is also responsible for the slow degradation of samples with very low DA. However, samples having a high DA showed a decreasing degradation rate with increasing DA (Fig. 2) relative to those samples with intermediate DA, which seems to contradict the theory that more *N*-acetyl groups results in faster degradation.

The enhanced degradation rate of samples with intermediate DAs can be explained by the solubility characteristics of chitosan in neutral aqueous environments. It is well known that chitosan is water-soluble when the DA is close to 50% [32,33]. Both the solubility and DA range of solubility increases with decreasing molar mass [34]. Thus, the solubility of low and medium molar mass fragments cleaved during degradation

should be significantly higher in samples having intermediate DAs. The enzymatic hydrolysis, which can be expected to increase with increasing DA, is therefore overshadowed by the enhanced solubility of the degrading macromolecule in samples with intermediate DAs, which results in their accelerated mass loss.

The specific degradation by lysozyme of chitosan and chitin can be beneficial in tissue engineering applications. Lysozyme is present in certain human body fluids [14,15,35]. In addition, the enzyme was reported to be synthesized during active phagocytosis after nerve injury [36]. Thus lysozyme, released from phagocytic cells including macrophages and neutrophils, will be available for the degradation of chitosan and chitin nerve guides.

### 3.3. DRG cell compatibility

Primary chick DRG neurons were cultured for 2 days on films of chitosan and *N*-acetylated chitosan in order to analyze the influence of the DA on the cell compatibility. The direct observation of cell adhesion and differentiation by light microscopy was facilitated by the transparency of the films. Based on the light microscopy photographs (Fig. 3) and the quantitative analysis (Figs. 4 and 5), all examined chitosan-based films, except those of intermediate DAs of 46.1% and 66.4%, supported DRG cell adhesion and neurite outgrowth.

The number of cells adhering to the materials was higher on chitosan derivatives having DAs of 0.5% and 10.9% than on materials with a DA of 37.5% and greater (Fig. 4). Samples with higher DAs showed cell clustering, which was particularly remarkable on films with DAs of 46.1% (Fig. 3b) and 66.4%, indicating a greater affinity of the cells for each other than for the materials surface. It was difficult to count the number of individual cells adhering to the film surfaces when cell clusters formed. For this reason, we also tested cell viability using the MTS assay. Cell viability decreased with increasing DA (Fig. 4), which reflects the need of DRG cell adhesion for viability and indicates that the cell clusters, which formed on samples with higher DAs, were filled with a large number of necrotic cells. For chitosan with a DA of 0.5%, cell viability was higher than that on all other materials. Remarkably, it was about 8 times higher than that on chitosan with a DA of 10.9%.

DRG neurite extension was assessed by analyzing the number of cells bearing neurites longer than one cell body length and the total neurite length (in NGF-supplemented media). Both parameters were generally seen to decrease with increasing DA of the samples (Fig. 5). No neurite outgrowth was observed on samples with DAs of 46.1% and 66.4%, which confirmed the poor cell compatibility observed by the formation of large cell clusters. The tendency for cluster formation and the resulting lower cell viability also limited neurite outgrowth on films with high DA.

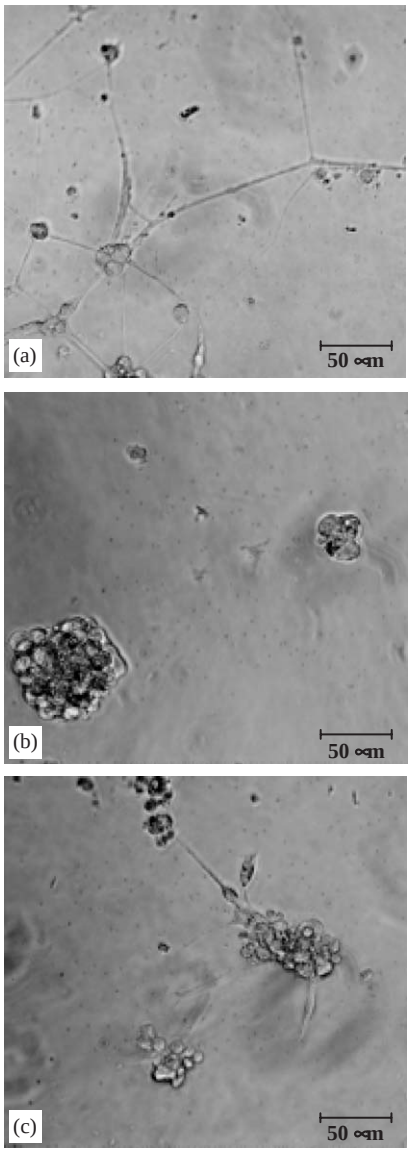


Fig. 3. DRG cell adhesion and neurite outgrowth after 2 days of culture on films of chitosan and *N*-acetylated chitosan ($1 \times 10^4$ cells/cm$^2$): (a) DA = 0.5%, (b) DA = 46.1% and (c) DA = 99.2%.

Previous studies have shown that chitosan promotes survival and neurite outgrowth of neural cells in vitro [9,37–42]. However, the influence of the DA on neural cell compatibility has not yet been described. DRG cell adhesion and neurite outgrowth on chitin/chitosan surfaces are affected by material characteristics, such as surface morphology, hydrophilicity and charge. Given that the difference between chitin and chitosan films is mostly amine content, it suggests that the presence of cationic ammonium charge accounts for the greater cell viability and neurite outgrowth on chitosan films having low DAs. The charge density of chitosan

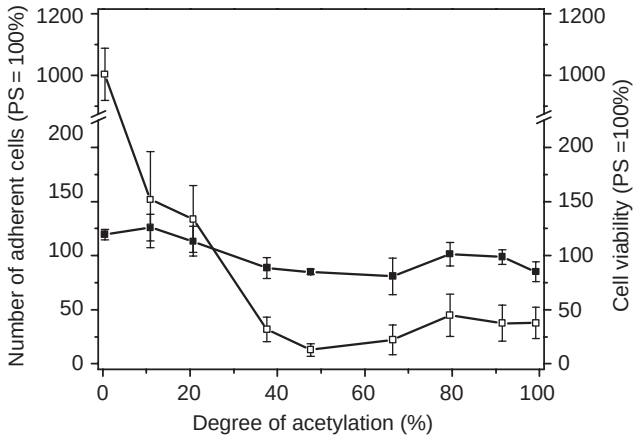


Fig. 4. Quantitative analysis of DRG cell adhesion (■, $n = 3$) and viability (□, $n = 5$) vs. DA after 2 days of culture on films of chitosan and *N*-acetylated chitosan. Data are shown as mean percentage ± standard deviation relative to tissue culture polystyrene controls (PS = 100%).

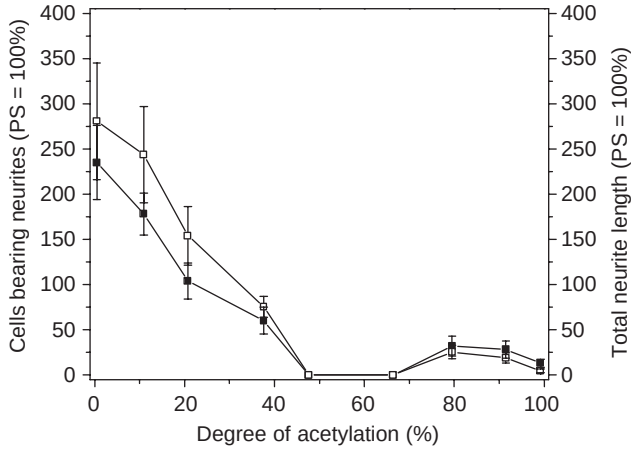


Fig. 5. Quantitative analysis of DRG neurite outgrowth vs. DA after 2 days of culture on films of chitosan and *N*-acetylated chitosan: number of cells bearing neurites (■, $n = 3$); total neurite length (□, $n = 3$). Data are shown as mean percentage ± standard deviation relative to tissue culture polystyrene controls (PS = 100%).

increases with decreasing DA and leads to greater cell adhesion. These findings with neural cell adhesion to chitosan are consistent with those of keratinocytes [10] and fibroblasts [10,23], suggesting a non-specific cell interaction between chitosan positively charged ammonium sites at physiological pH and negatively charged cell membrane surfaces [10]. The low cell viability and lack of neurite outgrowth observed for samples with intermediate DAs can be attributed to poor cell adhesion as these are anchorage-dependent cells, which is in turn affected by the lower concentration of amine groups and the weak and swollen surfaces of these films. It is worth noting that chitin films, providing a non-swollen surface for cell adhesion, allow for neurite outgrowth despite the very low amine content. Our results imply that cell compatibility can be adjusted by amine content, which is important for tissue engineering in the nervous system.

## 4. Conclusions

The DA of chitosan and chitin, which can be adjusted by controlled *N*-acetylation (or deacetylation) reactions, has a significant impact on the material properties important for tissue engineering applications, including biodegradation and biocompatibility. Prolonged degradation times and enhanced cell adhesion can be achieved using chitosan with a DA close to 0%. Slightly higher DAs, up to about 20%, allow for faster degradation and comparatively good cell adhesion and neurite outgrowth. Fastest degradation can be achieved with chitosan having intermediate DAs, but at the cost of limited cell adhesion. Modifying the DA of chitosan provides a powerful means for controlling biodegradation and biocompatibility and can be optimized for tissue engineering applications. In ongoing studies, we are investigating chitosan tubes for nerve regeneration and specifically the impact of acetylation on regenerative capacity.

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