Characterization of Chitosan and the Effects of Cleaning

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Statement of Purpose: Chitosan, a co-polymer of N-acetyl-glucosamine, is derived from chitin, a polysaccharide found in arthropods and other invertebrate tissue [1]. While there is much variation in processes, chitosan is generally obtained from shellfish shells, via soaking in strong alkali to remove residual animal tissues, then in strong acid to remove residual mineral, and then in hot alkali to remove N-acetyl side groups [1]. The percentage of N-acetyl groups removed, (i.e. degree of deacetylation, DDA) and the molecular weight (MW) are known to be important to chitosan properties [1]. However, amount of residual protein and mineral left in the chitosan after processing is generally unknown and may be important to physical and biological properties. This work evaluated the residual inorganic and proteineaous content of a commercial chitosan before and after a cleaning process and to evaluate changes in physical properties and in vitro cell responses.

Materials and Methods: A 78.7% DDA commercial chitosan (Vanson, Redmond, WA) was evaluated. DDA, MW, and residual ash content of the material were determined by titration, Gel Permeation Chromatography (GPC), and combustion respectively [2]. Residual protein was extracted from the chitosan via trichloroacetic acid/deoxycholate process and measured using the Pierce BCA Protein Assay. Then the chitosan was cleaned based on a chitin cleaning process as follows: dissolving chitosan in 2% acetic acid for 24 hours under stirring to remove residual ash, precipitation of chitosan with strong base, washing, then stirring in 5(w/v)% NaOH to remove residual protein material for 24 hours, washing again to neutral pH, and finally air drying in ambient conditions [1]. Once dried, the cleaned chitosan was retested for DDA, MW, residual inorganic and proteinaceous contents. An additional test for DDA was performed using Nuclear Magnetic Resonance (NMR). Two (w/v)% solution of the 'as received' and cleaned chitosan in 2% acetic acid were solution cast into thin films for water contact angle measurements and cell attachment. For the cell attachment, 3T3 murine fibroblasts were seeded at 10^4 cells/cm^2 onto films in 48 well plates. After 4 hours, non-adherent cells were removed counted and the number of attached cells determined by subtraction.

Results: Table lists the physical and chemical properties of 'as received' and cleaned chitosans. An error in the titration of the cleaned chitosan prevented DDA determination, so NMR was used to determine DDA. GPC analysis and NMR results demonstrated that the cleaning process did not change chitosan MW or DDA. With the cleaning process, there was a 6-fold decrease in residual ash and a 10-fold decrease in residual protein content. Statistical differences for residual protein content were not significant ($p=0.07$) in part due to the large standard deviations and small sample sizes, but the cleaned chitosan did show a significant increase in hydrophilic character as compared to the as received material ($p=0.006$). For the 4 hr cell attachment (Fig.) there was no difference between the chitosans ($p=0.2$).

Table: *Properties of 'As Received' & Cleaned Chitosan*

	As Received	Cleaned
DDA by titration (n=3)	76.5%±0.2%	---
DDA by NMR*	78.1%	77.7%
MW_n*	7.316 x 10^4 DA	7.461 x 10^4 DA
(MW_w)*	1.340 x 10^5 DA	1.257 x 10^5 DA
Ash Content *	3.47%	0.59%
Residual Protein (µg protein/ g chitosan) (n=3)	211.7 ±90_a	28.8 ±8_a
Contact Angle (n=3)	74.67°±3.46°$_a$	61.75°±2.45°$_b$

*n=1, subscript letters indicate statistical differences of $\alpha=0.05$

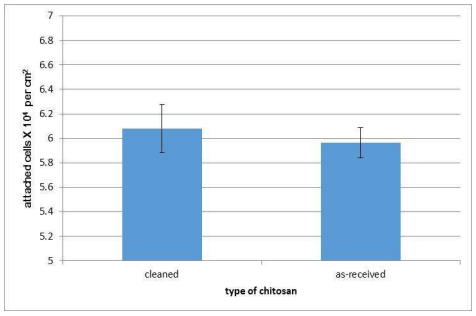

Figure: No. cells attached to the films at 4 hrs.

Conclusion: It is seen that the cleaning process did not affect MW or DDA. The cleaning process significantly increased the hydrophilic character of the chitosan presumably due to the removal of residual compounds though changes in residual contents were not significant. Additional measurements will provide clarification on these changes. While not significant, there was a trend of increased numbers of attached cells on the cleaned chitosan. Repeating the cleaning cycles may lead to larger changes in residual contents, that would have a more significant effects on properties and cells responses. These data suggest that residual compounds may be present in chitosans, that the levels of these compounds can influence physical properties and may influence cell responses, though additional testing is needed.

References:

1. Khor, E. *Chitin: Fulfilling a Biomaterials Promise.* Amsterdam: Elsevier Sci, 2001.
2.Yuan, et al. Materials 4 (2011): 1399-1406.

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