Carbohydrate Polymers 77 (2009) 420–424

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Effects of degree of deacetylation on enzyme immobilization in hydrophobically modified chitosan

Kyle H. Sjoholm [a], Michael Cooney [b], Shelley D. Minteer [a,*]

[a] Department of Chemistry, Saint Louis University, 3501 Laclede Ave., Saint Louis, MO 63103, USA
[b] Hawaii Natural Energy Institute, University of Hawaii-Manoa, Honolulu, HI, USA

ARTICLE INFO

Article history:
Received 30 September 2008
Received in revised form 28 January 2009
Accepted 9 February 2009
Available online 20 February 2009

Keywords:
Chitosan
Deacetylation
Reductive amination
Micellar polymers
Enzyme immobilization

ABSTRACT

Chitosan is a deacetylated form of the polysaccharide chitin. Over the last decade, researchers have employed reductive amination to hydrophobically modify chitosan to induce a micellar structure. These micellar polymers have been used for a variety of purposes including drug delivery and enzyme immobilization and stabilization. However, commercial sources of chitosan vary in their degree of deacetylation and there remains a paucity of information regarding how this can impact the modified polymer's functionality for enzyme immobilization. This paper, therefore, evaluates the effect that the degree of deacetylation has on the hydrophobic modification of medium molecular weight chitosan via reductive amination with long chain aldehydes and the resulting changes in enzyme activity after the immobilization of glucose oxidase in the micellar polymeric structure. The chitosan was deacetylated to differing degrees via autoclaving in 40–45% NaOH solutions and characterized using NMR, viscosity measurements, and differential scan calorimetry. Results suggest that a high degree of deacetylation provides optimal enzyme immobilization properties (i.e. high activity), but that the deacetylation method begins to significantly decrease the polymer molecular weight after a 20 min autoclave treatment, which negatively affects immobilized enzyme activity.

1. Introduction

Chitin, which can be found in the outer shells of crustaceans and insects, can be described as cellulose with one hydroxyl group on each monomer substituted with an acetylamine group. When the acetylamine group is replaced by an amine group, the linear biopolymer's solubility in aqueous solutions can increase. As shown in Fig. 1, this absence of acyl groups attached via amide bond to the polysaccharide backbone is the distinguishing difference between chitosan and chitin, with the transition from chitin to chitosan generally assumed to occur when the fraction of acetylated amine groups dips below 35–40% (Kristbergsson, Einarsson, Hauksson, Peter, & Gislason, 2003). Since chitin is the second most abundant biomass next to cellulose, the more soluble chitosan has naturally become a material of increased interest due to its biocompatibility, biodegradability, chemical inertness, high mechanical strength, and non-toxic properties (Klotzbach, Watt, Ansari, & Minteer, 2006). For example, chitosan has been proposed as a potential alternative to Nafion and other polyelectrolytes in the chemical modification of electrodes. Chitosan has also often been chemically modified to produce a polymer of altered functionality. However, the chemical and physical properties of the modified chitosan can vary as a function of the degree to which the polymer was deacetylated (Basavaraju, Damappa, & Rai, 2006; Chandumpai, Singhpibulporn, Faroongsarng, & Sornprasit, 2004; Huang, Khor, & Lim, 2004; Wang et al., 2006; Zhou et al., 2008).

One important modification has been the chemical modification of chitosan via the amine groups for applications in drug delivery, gene delivery, and enzyme immobilization and stabilization (Esquenet & Buhler, 2001; Esquenet, Terech, & Boue, 2004; Klotzbach et al., 2006; Lee, Gustin, Chen, Payne, & Raghavan, 2005). In particular, hydrophobic modification of the hydrophilic chitosan backbone allows for the formation of micellar structures. Micellar structures are attractive for drug delivery and gene delivery, because they allow for delivery of drugs and genes within matrices where solubility is an issue (Eiyahu et al., 2005; Feng, Wang, Lin, & Xu, 2006; Rangel-Yagui, Pessoa, & Travares, 2005; Torchilin, 2007; Zhang, Ding, Yu, & Ping, 2007; Zhu, Zhang, Wu, Tang, & Ping, 2008). Micellar structures have also been shown to be attractive for enzyme encapsulation, often increasing thermal tolerance, chemical tolerance, and long term stability (Bru, Sanchez-Ferrer, & Garcia-Carmona, 1995; Callahan & Kosicki, 1967; Celej, D'Andrea, Campana, Fidelio, & Bianconi, 2004; Galarneau et al., 2007; Gebicka & Jurgas-Grudzinska, 2004; Kimura et al., 2004).

Because the degree of deacetylation of chitosan is a function of both the supplier and the batch, the modified polymer's functionality can also vary. To explore this effect, this work details the ef-

* Corresponding author. Tel.: +1 314 977 3624; fax: +1 314 977 2521.
E-mail address: minteers@slu.edu (S.D. Minteer).

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.02.006

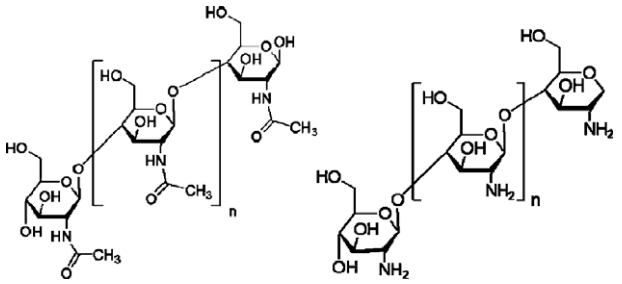

Fig. 1. Structures of (Left) chitin and (Right) fully deacylated chitosan.

fects of degree of deacetylation of chitosan on the hydrophobic modification of chitosan and the resulting ability of the modified chitosan to immobilize enzymes. By employing alkaline treatment to deacetylate commercially available medium molecular weight chitosan via autoclaving, the effects of deacylation have been characterized using NMR, differential scan calorimetry, and viscosity measurements, which are traditional techniques for characterizing these polymers. The deacylated chitosan was then hydrophobically modified with butyraldehyde via reductive amination and the chemical and physical properties of the hydrophobically modified chitosan characterized as a function of the degree of deacetylation before enzyme immobilization. A comparison of immobilized enzyme activity as a function of degree of deacetylation was performed with glucose oxidase to show that the degree of deacetylation does indeed affect enzyme activity after hydrophobic modification of the chitosan. This investigation demonstrated the power of deacetylation on the chitosan's ability to provide as an optimal chemical microenvironment for enzyme encapsulation for use in biofuel cells, sensors, and biocatalysis.

2. Experimental

2.1. Reagents

Medium molecular weight chitosan (Aldrich), glucose oxidase (Sigma), glucose (Sigma), 98% NaOH pellets (Aldrich), peroxidase (Sigma), sodium cyanoborohydride (Aldrich), butanal (Aldrich), o-dianisidine (Sigma), glacial acetic acid (Fisher), deuterated acetic acid 99.5% (Aldrich), deuterium oxide 99.9% (Aldrich) were used without further purification.

2.2. Deacetylation of chitosan by autoclaving

Chitin was deacetylated according to the procedure of No, Cho, Kim, & Meyers, 2000. Medium molecular chitosan was combined with 40–45% NaOH solution in a 1:15 solid to liquid ratio to a final volume of 100 mL. The mixture was then autoclaved (Harvey Sterilemax) at 121 °C and 30 psi for periods of 10, 20, and 30 min. The autoclave was allowed to go through its temperature cycle and then removed from the autoclave. The mixture was then allowed to cool to room temperature and then vacuum filtered using P5 filter paper (Fisher Brand) and washed with 18 MΩ water until neutralized. Air was pulled through the sample until dry. The filtrate was then dried over dessicant for 24 h. All experiments were performed in triplicate.

2.3. NMR sample preparation analysis

To ensure elimination of water, 0.05 g portions of chitosan were placed in vials and heated in a vacuum oven at 50 °C for 1 h. The vials were then capped and allowed to cool to room temperature before being filled with 2 mL of 1% deuterated acetic acid solution. The vials were then placed on a vortex for 1 h to thoroughly mix and solvate the chitosan. 128 scans of ^{1}H NMR were collected for each sample using a Bruker 400 MHz NMR spectrometer.

2.4. Hydrophobic modification of chitosan

A suspension was made with 0.500 g of medium molecular weight chitosan in 30 mL of a 1:1 mixture of 1% (vol.) acetic acid and pure methanol with continuous stirring for 10 min. To this mixture, 20 mL of butyraldehyde was added along with 0.7 g of sodium cyanoborohydride. After 10 min of additional stirring, the white precipitate was vacuum filtered and rinsed thoroughly with methanol being dried under desiccation. The dried chitosan was then re-suspended in a 1% (w/v) of 1% acetic acid solution or *t*-amyl alcohol.

2.5. Viscosity measurements

Kinematic viscosity is measured directly from the viscometer readings and is a measure of the absolute viscosity divided by the density of the solution. Seven milliliters of 0.5% (w/v) chitosan suspensions were prepared in 3% (v/v) acetic acid solution. 6.7 mL of each chitosan suspension was transferred to a Cannon–Fenske Routine type viscometer for transparent liquids (No. 200 S57). Times were measured using a Timex Ironman stopwatch and recorded in triplicate. The kinematic viscosity was calculated using the times and the instrumental constant (0.920 mm^2/s^2). The viscosity was determined by multiplying the kinematic viscosity by the density (g/mL) that was determined for each of the polymers. All measurements were done in triplicate and reported errors correspond to one standard deviation.

2.6. Differential scan calorimetry

Samples were weighed and added to aluminum pans. Temperatures were scanned from 60 to 220 °C at scan rates of 10 °C/min on a Perkin–Elmer DSC with thermal control using Pyris Series Software while flowing N_2 gas over the sample at 20 mL/min. All measurements were done in triplicate and reported errors to the glass transition temperature (T_g) corresponded to one standard deviation.

2.7. Glucose oxidase activity assays

Activity assays were executed on glucose oxidase immobilized on the bottom of 1.5 mL disposable cuvettes (Fisher Scientific) within hydrophobically modified chitosan of varying degrees of deacetylation. Fifty microliters of glucose oxidase suspended in sodium acetate buffer (2 U/mL) was mixed with 50 μL of 1 wt% chitosan suspension dissolved in 1% acetic acid. Twenty-five microliters of this mixture was then cast into the bottom of the cuvette and allowed to dry in a vacuum desiccator. 1.4 mL of a reaction cocktail consisting of 10% (w/v) β-D-glucose solution and 0.17 mM o-dianisidine was added to the cuvette. Hundred microliters peroxidase solution (POD, 60 U/mL in cold deionized water) was then added and the change in absorbance was recorded with time. Activities per gram were then determined from the change in absorbance (ΔA) per minute.

3. Results and discussion

Heterogeneous deacetylation was performed by autoclaving chitosan in the presence of an alkaline medium (NaOH). It is important to note that chitosan is not really soluble in NaOH, so when chitosan was treated with NaOH, it formed a discrete layer

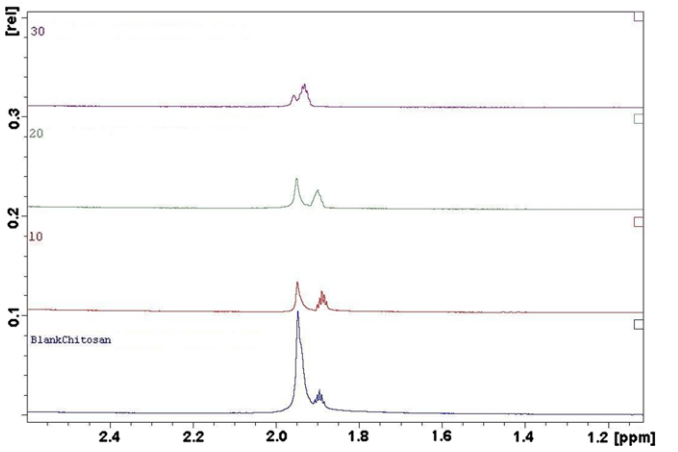

Fig. 2. ^{1}H NMR spectra of as received chitosan (bottom), of chitosan following 10 min of treatment (2nd from bottom), of chitosan following 20 min of treatment (3rd from bottom), of chitosan following 30 min of treatment (top), showing that increased treatment time results in greater deacetylation of chitosan over time making the acyl peaks smaller.

between the NaOH solution and the solvated chitosan. Upon completion of treatment in the autoclave, the mixture was more homogeneous and an orange tinted solution, with the color increasing in intensity with heating time. Upon drying, the chitosan was a fine ecru powder which was similar in texture to the untreated chitosan.

^{1}H NMR spectroscopy was performed on each sample and the peak at 1.9 ppm, corresponding to the methyl protons of the acyl amide, demonstrated a decreased intensity relative to the other peaks, suggesting that the longer NaOH treatments resulted in more deacetylation, as shown in Fig. 2 (Sashiwa, Yajima, & Aiba, 2003). The quintet to the right of this peak, which corresponds to the solvent, remained equal in intensity throughout all experiments. The 80% deacetylated commercial chitosan was 93% deacetylated after 10 min of alkaline treatment via autoclave, as determine by ^{1}H NMR measurement. After 20 min of alkaline treatment via autoclave, the chitosan polymer was found to be 94% deacetylated, and after 30 min the polymer was found to be 98% deacetylated.

Table 1 shows the results of the viscosity measurements of 0.5% (w/v) chitosan solutions in 3% by volume acetic acid. Both absolute viscosity and kinematic viscosity decreased with increased degree of deacylation up to 30 min of the autoclave treatment. This agrees with theoretical prediction of a decrease in viscosity with increasing degree of deacetylation due to more amine functional groups being available for protonation and therefore increased solubility.

Table 1
Viscosity and kinematic viscosity of 0.5% chitosan and hydrophobically modified chitosan solutions in acetic acid after different deacetylation reaction times.

Polymer	Kinematic viscosity (cSt)	Viscosity (cP)	Intrinsic viscosity (dl/g)
Chitosan – 0 min	25.09 ± 0.03	30.45 ± 0.04	38.45
Chitosan – 10 min	17.84 ± 0.06	21.45 ± 0.07	40.83
Chitosan – 20 min	10.50 ± 0.04	12.67 ± 0.04	26.78
Chitosan – 30 min	15.96 ± 0.06	19.26 ± 0.07	19.14
Hydrophobically Modified chitosan – 0 min	9.39 ± 0.02	15.26 ± 0.04	17.17
Hydrophobically modified chitosan – 10 min	8.39 ± 0.08	10.07 ± 0.10	20.14
Hydrophobically modified chitosan – 20 min	8.61 ± 0.02	10.38 ± 0.02	19.83
Hydrophobically modified chitosan – 30 min	8.13 ± 0.02	9.80 ± 0.02	19.46

Table 2
Molecular weight of deacetylated chitosans.

Polymer	Molecular weight (g/mol)
Chitosan – 0 min	3,363,504
Chitosan – 10 min	3,660,416
Chitosan – 20 min	2,021,071
Chitosan – 30 min	1,259,573

Intrinsic viscosity, the ratio of the solution viscosity to the concentration of the solute extrapolated to zero concentration, decreased with an increase in degree of deacetylation across all samples. This corresponds with previous literature where chitosan that had been deacetylated in a different fashion was found to have an inverse relationship between deacetylation and viscosity (Chandumpai et al., 2004). By eliminating acetyl groups from the polymer backbone, the hydrophobicity of chitosan chain is reduced causing the viscosity to decrease. However, the observed change in viscosity and kinematic viscosity with degree of deacetylation was less pronounced for the hydrophobically modified chitosan produced via reductive amination with butyraldehyde. This is most likely due the degree of substitution of butyl groups onto the amine group of chitosan.

Intrinsic viscosity can also be employed to determine the molecular weight of chitosan polymers. Table 2 shows the molecular weights of each of the alkaline treated chitosans calculated from the Mark–Houwink equation using the intrinsic viscosity data extrapolated to zero concentration. Previous research suggests that doubling reaction time should decrease molecular weight by 20–30% (Methacanon, Prasitsilp, Pothsree, & Pattaraarchachai, 2003). In our work, we found this to be true for autoclave treatments from 10 to 20 min, but that longer treatment time resulted in a substantial breakdown of the polymeric backbone, yielding a significant decrease in the polymer's molecular weight.

Differential scanning calorimetry was used to investigate the change in glass transition temperature (T_g) for chitosan polymers as a function of degree of deacetylation and hydrophobic modification. Table 3 shows the glass transition temperature for chitosan polymers of different preparations. It can be shown that glass transition temperatures decreased with an increase in degree of deacetylation until the breakdown of the chitosan backbone structure (30 min). This corresponds with other literature reports for how degree of deacetylation affects the glass transition temperature of chitosan (Dong, Ruan, Wang, Zhao, & Bi, 2004). It was also shown that glass transition temperature decreased with hydrophobic modification. Although not reported previously, this result is consistent with measurements made on hydrophobically modified Nafion that have shown its glass transition temperature decreased when hydrophobically modified with tetralkylammonium cations (Page, Cable, & Moore, 2005). This is of particular importance for enzyme immobilization, because many applications require heat treatments.

Previous research has shown that hydrophobically modified chitosan provides an optimal microenvironment for enzyme immobilization (Klotzbach, Watt, Ansari, & Minteer, 2008; Klotzbach et al., 2006) and that the microenvironment can be tailored

Table 3
Glass transition temperature for different chitosans.

Polymer	Glass transition temperature (°C)
Chitosan – 0 min	143.2 ± 1.0
Chitosan – 10 min	130.4 ± 3.1
Chitosan – 20 min	124.4 ± 0.1
Chitosan – 30 min	135.7 ± 0.4
Hydrophobically modified chitosan – 0 min	117.3 ± 1.1

Table 4
Activity of glucose oxidase in hydrophobically modified chitosan compared to glucose oxidase in solution.

Polymer	Activity (Units/g)
Chitosan – 0 min	608 ± 149
Chitosan – 10 min	876 ± 60
Chitosan – 20 min	1207 ± 419
Chitosan – 30 min	86 ± 9
Enzyme in buffer	5509 ± 63

by altering the hydrophobic alkyl chain (butyl, hexyl, octyl, decyl) (Klotzbach et al., 2008). A variety of oxidoreductase enzymes, including: alcohol dehydrogenase, formate dehydrogenase, glucose dehydrogenase, lactic dehydrogenase, and glucose oxidase, have been studied. To investigate the effects of degree of deacetylation on enzymatic activity, glucose oxidase was immobilized in hydrophobically modified chitosan and spectrophotometric enzymatic activity assays were performed. Butyl modified chitosan was employed for all of these studies, because butyl modified chitosan is soluble regardless of the degree of deacetylation, so a complete study can be accomplished. Table 4 shows the activity of glucose oxidase immobilized in hydrophobically modified chitosan with different degrees of deacetylation. It is important to note that one Unit of activity corresponds to the ability of glucose oxidase to oxidize one micromole of glucose per minute per milligram. Although the enzyme activity across all samples was less when immobilized (as opposed to free enzyme in buffer) due to decreased mass transport of substrate within the polymer, the enzyme activity of immobilized enzyme was found to increase with increased degree of deacetylation until the breakdown of the chitosan backbone structure (30 min), which resulted in more than an order of magnitude (14-fold) decrease in enzyme activity. Increased degree of deacetylation from 80% to 94% without significant decrease in molecular weight resulted in a 2-fold increase in enzyme activity for glucose oxidase immobilized in the resulting hydrophobically modified chitosan. This is extremely significant for biosensing, biofuel cells, or bioprocessing applications, because it results in a 2-fold increase in sensitivity, power, or efficiency, respectively. Therefore, this research has shown that it is important for enzyme immobilization applications to work with chitosans with a high (>90%) degree of deacetylation, but that researchers need to focus special attention on the deacetylation procedure to ensure that it is not breaking down the chitosan backbone structure which would result in significant decreases in enzymatic activity.

4. Conclusions

Although chitosan polymers with different degrees of deacetylation can all be hydrophobically modified via reductive amination with long chain aldehydes, this paper has shown that the physical and chemical properties of the hydrophobically modified chitosan is dependent upon the degree of deacetylation of the original chitosan reactant polymer. Consequently, the viscosity, molecular weight, degree of deacetylation and degree of hydrophobic modification of chitosan need to be optimized for the use of hydrophobically modified chitosan for enzyme immobilization. This paper has also shown that alkaline treatment via heat treatment can deacetylate commercially available chitosan previous to hydrophobic modification without major changes in polymer molecular weight as long as treatment is no longer than 20 min. The paper has shown that this greater degree of deacetylation results in a 2-fold increase in enzyme activity for glucose oxidase immobilized in the resulting hydrophobically modified chitosan. However, heat treatment for longer than 20 min results in a significant decrease in polymer molecular weight (63% for 30 min) and intrinsic viscosity and an increase in glass transition temperature, while not significantly changing the degree of deacetylation (degree of deacetylation goes from 94% after 20 min to 98% after 30 min). This change in the physical and chemical properties of chitosan after long heat treatment resulted in a 14-fold decrease in enzyme activity. Therefore, autoclaving procedures should be optimized to maximize degree of deacetylation while not significantly decreasing molecular weight for producing a chitosan polymer that can then be hydrophobically modified and employed for enzyme immobilization.

Acknowledgements

The author of the paper would like to thank the Air Force Office of Scientific Research and Akermin Inc., for their generous support and funding, as well as. Dr. James Chickos from University of Missouri – St. Louis for use of his differential scan calorimeter.

References

Basavaraju, K. C., Damappa, T., & Rai, S. K. (2006). Preparation of chitosan and its miscibility studies with gelatin using viscosity, ultrasonic and refractive index. *Carbohydrate Polymer, 66*, 357–362.
Bru, R., Sanchez-Ferrer, A., & Garcia-Carmona, F. (1995). Kinetic models in reverse micelles. *Biochemical Journal, 310*, 721–739.
Callahan, J. W., & Kosicki, G. W. (1967). The effect of lipid micelles on mitochondrial malate dehydrogenase. *Canadian Journal of biochemistry, 45*, 839–851.
Celej, M. S., D'Andrea, M. G., Campana, P. T., Fidelio, G. D., & Bianconi, M. L. (2004). Superactivity and conformational changes on chymotrypsin upon interfacial binding to cationic micelles. *Biochemical Journal, 378*, 1059–1066.
Chandumpai, A., Singhpibulporn, N., Faroongsarng, D., & Sornprasit, P. (2004). Preparation and physico-chemical characterization of chitin and chitosan from the pens of the squid species, *Loligo lessoniana* and *Loligo formosana*. *Carbohydrate Polymer, 58*, 467–474.
Dong, Y., Ruan, Y., Wang, H., Zhao, Y., & Bi, D. (2004). Studies of glass transition temperature of chitosan with four techniques. *Journal of Applied Polymer Science, 93*, 1553–1558.
Eiyahu, H., Makovitzki, A., Azzam, T., Zlotkin, A., Joseph, A., Gazit, D., et al. (2005). Novel dextran–spermine conjugates as transfecting agents: Comparing water-soluble and micellar polymers. *Gene Therapy, 12*(6), 494–503.
Esquenet, C., & Buhler, E. (2001). Phase behavior of associating polyelectrolyte polysaccharides. 1. Aggregation process in dilute solution. *Macromolecules, 34*(15), 5287–5294.
Esquenet, C., Terech, P., & Boue, F. (2004). Structural and rheological properties of hydrophobically modified polysaccharide associative networks. *Langmuir, 20*(9), 3583.
Feng, X., Wang, C., Lin, B., & Xu, F. (2006). Methoxy poly(ethylene glycerol)-conjugated linoleic acid polymeric miceless for paclitaxel delivery. *Colloid Journal, 68*(6), 779–783.
Galarneau, A., Renard, G., Mureseanu, M., Tourrette, A., Biolley, C., Choi, M., et al. (2007). Synthesis of sponge mesoporous silicas from lecithin/dodecylamine mixed-micelles in ethanol/water media: A route towards efficient biocatalysts. *Microporous and Mesoporous Materials, 104*, 103–114.
Gebicka, L., & Jurgas-Grudzinska, M. (2004). Activity and stability of catalase in nonionic micellar and reverse miceller systems. *Zeitschrift fur Naturforschung, 59c*, 887–891.
Huang, M., Khor, E., & Lim, L. Y. (2004). Uptake and cytotoxicity of chitosan molecules and nanoparticles: Effects of molecular weight and degree of deacetylation. *Pharmaceutical Research, 21*(2), 344–353.
Kimura, M., Michizoe, J., Oakazaki, S. Y., Furusaki, S., Goto, M., Tanaka, H., et al. (2004). Activation of lignin peroxidase in organic media by reversed micelles. *Biotechnology and Bioengineering, 88*(4), 495–501.
Klotzbach, T. L., Watt, M. M., Ansari, Y., & Minteer, S. D. (2006). Effects of hydrophobic modification of chitosan and Nafion on transport properties, ion-exchange capacities, and enzyme immobilization. *Journal of Membrane Science, 282*(1), 276–283.
Klotzbach, T. L., Watt, M. M., Ansari, Y., & Minteer, S. D. (2008). Improving the microenvironment for enzyme immobilization at electrodes by hydrophobically modifying chitosan and Nafion polymers. *Journal of Membrane Science, 311*, 81–88.
Kristbergsson, K., Einarsson, J., Hauksson, S., Peter, K., & Gislason, J. (2003). Recent developments in deacetylation of chitin, and possible applications in food formulation. Trans Atlantic fisheries technology. Iceland: Reykjavik.
Lee, J. H., Gustin, J. P., Chen, T., Payne, G. F., & Raghavan, S. (2005). Vesicle-biopolymer gels: Networks of surfactant vesicles connected by associating biopolymers. *Langmuir, 21*(1), 26–33.
Methacanon, P., Prasitsilp, M., Pothsree, T., & Pattaraarchachai, J. (2003). Heterogeneous N-deacetylation of squid chitin in alkaline solution. *Carbohydrate Polymer, 52*, 119–123.
No, H. K., Cho, Y. I., Kim, H. R., & Meyers, S. P. (2000). Effective deacetylation of chitin under conditions of 15 psi/121 °C. *Journal of Agricultural and Food Chemistry, 48*(6), 2625–2627.

Page, D. A., Cable, K. M., & Moore, R. B. (2005). Molecular origins of the thermal transitions and dynamic mechanical relaxations in perfluorosulfonate ionomers. *Macromolecules, 38*, 6472–6484.

Rangel-Yagui, C. O., Pessoa, A., & Travares, L. C. (2005). Micellar solubilization of drugs. *Pharmacy and Pharmaceutical Sciences, 8*(2), 147–163.

Sashiwa, H., Yajima, H., & Aiba, S.-i. (2003). Synthesis of a chitosan–dendrimer hybrid and its biodegradation. *Biomacromolecules, 4*(5), 1244–1249.

Torchilin, V. P. (2007). Micellar nanocarriers: Pharmaceutical perspectives. *Pharmaceutical Research, 24*(1), 1–16.

Wang, Q. Z., Chen, X. G., Liu, N., Wang, S. X., Liu, C. S., Meng, X. H., et al. (2006). Protonation constants of chitosan with different molecular weight and degree of deacetylation. *Carbohydrate Polymer, 65*, 194–201.

Zhang, C., Ding, Y., Yu, L., & Ping, Q. (2007). Polymeric micelle systems of hydroxycamptothecin based on amphiphilic N-alkyl-N-trimethyl chitosan derivatives. *Colloids and Surfaces B: Biointerfaces, 55*(2), 192–199.

Zhou, H. Y., Chen, X. G., Kong, M., Liu, C. S., Cha, D. S., & Kennedy, J. F. (2008). Effect of molecular weight and degree of chitosan deacetylation on the preparation and characteristics of chitosan thermosensitive hydrogel as a delivery system. *Carbohydrate Polymer, 73*(2), 265–273.

Zhu, X., Zhang, C., Wu, X., Tang, X., & Ping, Q. (2008). Preparation, physical properties, and stability of gambogic acid-loaded micelles based on chitosan derivatives. *Drug Development and Industrial Pharmacy, 34*(1), 2–9.