Published in final edited form as:

Carbohydr Polym. 2016 August 1; 146: 420–426. doi:10.1016/j.carbpol.2016.03.003.

Formulation and Characterization of a Plasma Sterilized, Pharmaceutical Grade Chitosan Powder

Andrew R Crofton^{a,c}, Samuel M Hudson^d, Kristy Howard^c, Tyler Pender^c, Abdelrahman Abdelgawad^d, Daniel Wolski^d, and Wolff M Kirsch^{*,b,c}

Andrew R Crofton: acrofton@llu.edu; Samuel M Hudson: shudson@ncsu.edu; Kristy Howard: khoward@llu.edu; Tyler Pender: tpender@llu.edu; Abdelrahman Abdelgawad: aabdelg@ncsu.edu; Daniel Wolski: dwolski@ncsu.edu; Wolff M Kirsch: wkirsch@llu.edu

^aDepartment of Anatomy, Loma Linda University, 11234 Anderson St., Medical Center A537, Loma Linda, CA 92350

^bDivision of Biochemistry, Loma Linda University, 11234 Anderson St., Medical Center A537, Loma Linda, CA 92350

^cNeurosurgery Center for Research, Training, and Education, School of Medicine, Loma Linda University, 11234 Anderson St., Medical Center A537, Loma Linda, CA 92350

^dCollege of Textiles, North Carolina State University 2401 Research Dr. Raleigh, North Carolina 27695

Abstract

Chitosan has great potential as a pharmaceutical excipient. In this study, chitosan flake was micronized using cryo-ball and cryo-jet milling and subsequently sterilized with nitrogen plasma. Micronized chitosan was characterized by laser diffraction, scanning electron microscopy (SEM), conductometric titration, viscometry, loss on drying, FTIR, and limulus amoebocyte lysate (LAL) assays. Cryo-jet milling produced mean particle size of 16.05 μm , 44% smaller than cryo-ball milling. Cryomilled chitosan demonstrated increased hygroscopicity, but reduced molecular weight and degree of deacetylation (DD). SEM imaging showed highly irregular shapes. FTIR showed changes consistent with reduced DD and an unexplained shift at 1100 cm^{-1} . Plasma treated chitosan was sterile with <2.5 EU/g after low-pressure plasma and <1.3 EU/g after atmospheric pressure plasma treatment. Plasma treatment decreased the reduced viscosity of chitosan flake and powder, with a greater effect on powder. In conclusion, pharmaceutical grade, sterile chitosan powder was produced with cryo-jet milling and plasma sterilization.

Keywords

Chitosan; Cryomilling; Jet Milling; Ball Milling; Cryo-ball Milling; Cryo-jet milling; Plasma Sterilization

*Corresponding Author: +1-909-558-7071, fax: +1-909-558-0472, wkirsch@llu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

Chitosan (CS) in the presence of moisture is a soft, ductile biopolymer with great potential as a biomedical material, especially as an excipient and immune adjuvant. CS is obtained commercially by deacetylating chitin extracted from either crustacean shells via chemical extraction or fungi via enzymatic extraction and consists of β -1,4-linked 2-amino-2-deoxy-D-glucopyranose and N-acetamido-2-deoxy-D-glucopyranose moieties that are randomly distributed throughout the polymer chain. Since chitin is the second most abundant biopolymer on Earth and crustacean shell waste generated by the seafood industry is an environmental problem in coastal areas, CS is a widely available, green, and economical biomaterial. Chitin becomes CS once at least 50% of the D-glucosamine moieties in the polymer chain are deacetylated (Pillai, Paul & Sharma, 2009). However, there is disagreement over the naming of chitin and CS based on degree of deacetylation, which has resulted in a proposal for naming chitin and CS based on solubility in acetic acid (Badawy & Rabea, 2011; Kumar, 2000). CS is soluble in aqueous acetic acid, whereas chitin is not.

As an excipient, CS enhances drug penetration through tissues and epithelial barriers by loosening gap junctions, maintains drug in the area of interest through bioadhesion between cationic amino groups of CS and anionic tissues, and controls drug release over time by keeping drugs bound until physical degradation (Artursson, Lindmark, Davis & Illum, 1994; Dodane, Amin Khan & Merwin, 1999; Jameela, Misra & Jayakrishnan, 1994; Kristl, Šmid-Korbar, Štruc, Schara & Rupprecht, 1993; Luessen, de Leeuw, Langemeyer, de Boer, Verhoef & Junginger, 1996; Nordtveit, Vårum & Smidsrød, 1994; Schipper, Vårum & Artursson, 1996; Takeuchi, Yamamoto, Niwa, Hino & Kawashima, 1996; van der Lubben, Verhoef, van Aelst, Borchard & Junginger, 2001). The biological functions of CS are dependent on its physical properties, including molecular weight (MW), degree of deacetylation (DD), salt form, and pH at which it is used (Dodane & Vilivalam, 1998; Kumirska, Weinhold, Thöming & Stepnowski, 2011). This physical-functional relationship necessitates careful characterization of CS formulations. Since powder and hydrogel forms of CS – especially those composed of particles in the low micron range – have been identified as the ideal form of CS for pharmaceutical applications and micronization of materials is a common process in pharmaceutical manufacturing, the goal of the present study was to formulate and characterize a pharmaceutical grade CS powder that can also serve as a precursor to a hydrogel when dissolved in dilute acids (van der Lubben, Verhoef, van Aelst, Borchard & Junginger, 2001).

A powder form of CS was also desired to enhance the effectiveness of a novel sterilization method for CS based on non-thermal nitrogen gas plasma (NtNP) (de Oliveira Cardoso Macedo, de Macedo, Gomes, de Freitas Daudt, Rocha & Alves, 2013). Plasma is considered the fourth state of matter and consists of an ionized gas that has emergent properties the gas alone does not, such as magnetism and conductivity. Use of NtNP for sterilization of CS is necessitated by the fact that conventional sterilization methods like dry/wet heat, radiation, and chemical sterilants cause caramelization of the polysaccharide, chain scissions, and/or may leave residual toxic residues in the material (de Oliveira Cardoso Macedo, de Macedo, Gomes, de Freitas Daudt, Rocha & Alves, 2013; Franca et al., 2013; Lim, Khor & Koo, 1998; Lim, Khor & Ling, 1999; Marreco, da Luz Moreira, Genari & Moraes, 2004; Norzita,

Norhashidah, Maznah, Nurul Aizam Idayu Mat, Nor Akma & Norafifah Ahmad, 2013; Rao & Sharma, 1995; Rosiak, Ula ski, Kucharska, Dutkiewicz & Judkiewicz, 1992; San Juan et al., 2012). These physicochemical changes result in changes in the biological properties of CS making these methods non-optimal for sterilizing CS. Since NtNP is a surface treatment, it benefits from a large surface to volume ratio, which is accomplished when a material is micronized.

Knowledge on micronizing hard and crystalline materials is extensive, but lacking on softer materials. It is vital to characterize particle size and size distribution of powders since these properties influence flowability, dissolution, release kinetics, and more (Koennings, Sapin, Blunk, Menei & Goepferich, 2007; Miranda, Millán & Caraballo, 2007; Mullarney & Leyva, 2009). Reports in the literature of techniques for generating micronized CS powders are especially sparse. Techniques for micronizing CS that have been reported include dense gas anti-solvent precipitation, supercritical-assisted atomization, microsphere precipitation, high speed planetary ball mill, and ultrafine milling (Chien, Li, Lee & Chen, 2013; Gimeno, Ventosa, Boumghar, Fournier, Boucher & Veciana, 2006; Reverchon & Antonacci, 2006; Yao, Peng, Yin, Xu & Goosen, 1995; Zhang, Zhang, Jiang & Xia, 2012; Zhang, Zhang & Xia, 2014). We identified cryomilling as an optimal method for generating a CS powder based on previous studies that show it preserves functional properties of proteins and starches by reducing the energy input needed to fractionate particles into smaller sizes (Dhital, Shrestha, Flanagan, Hasjim & Gidley, 2011; Ehmer, 2010; Tran, Shelat, Tang, Li, Gilbert & Hasjim, 2011). Additionally, cryomilling overcomes the soft ductile nature of CS, which reduces the effectiveness of traditional milling techniques in micronizing soft materials like CS (Garmise et al., 2006; Saleem & Smyth, 2010).

In the present study, two cryomilling techniques were tested, cryo-jet and cryo-ball milling. Ball and jet mills were chosen for this study since they are the only milling machines commonly used to reduce particles to 5 μm or less in dry conditions. (Vatsaraj et al 2003) Both ball and jet mills are thought to reduce particle sizes using the same mechanism(s), which is by breaking particles along cracks or fractures that already exist at the micro- or nanoscale. Although jet milling is the most commonly used technique for producing particles in the lower micrometer range and is the gold standard for manufacture of inhalable particles of small molecular drugs, this study is the first reported use of cryo-jet milling for micronizing CS (Ehmer, 2010). Once the optimal CS powder formulation was identified, defined as the micronized powder with the smallest mean particle size, it was sterilized with NtNP to form a sterile, pharmaceutical grade CS powder. Physicochemical properties of the CS were characterized before and after both milling and NtNP sterilization.

2. MATERIALS AND METHODS

2.1 Reagents

All chemical reagents including glacial acetic acid, lactic acid, sodium acetate, hydrochloric acid, sodium hydroxide, and tryptic soy agar were obtained from Sigma Aldrich or Fisher Scientific and were of analytical grade.

2.2 Cryomilling

CS derived from crab shells in the form of 1–10 mm flakes was obtained from Scion Biomedical, Inc. (Miami, FL). CS flakes were filtered with fine mesh to remove flakes > 8 mm in diameter and approximately 30 g of filtered CS flake was placed in a 25 mL zirconium oxide jar. Six milling balls made of either zirconium oxide (10 mm diameter) or stainless steel (5 mm) were added to the jar and the CS flakes were subsequently milled under cooling with liquid nitrogen at 30 Hz for up to 30 minutes in the Retsch CryoMill system (Verder Scientific, Inc., Newtown, PA). For cryo-jet milling, CS flakes were filtered, as described above, to remove flakes > 8 mm, 470 g of filtered CS was placed in the Micron-Master jet pulverization system (The Jet Pulverization Co., Inc., Moorestown, NJ), cooled with liquid nitrogen, and milled with a jet stream of liquid nitrogen for 30 minutes.

2.3 Particle Sizing

Particle size analyses were performed on powders by suspending CS in water and measuring particle size with a Horiba LA-930 laser diffraction analyzer (HORIBA Instruments, Inc., Irvine, CA).

2.4 Degree of Deacetylation

Degree of deacetylation (DD) of both CS flake and powder was determined by conductometric titration. Conductometric titration was performed by dissolving dried CS sample of known mass (about 0.100 g) into 10 mL of 0.1 N hydrochloric acid (HCl) then 90 mL of distilled water. The CS solution was then titrated with a standard 0.1 N sodium hydroxide (NaOH) solution using a 10 mL buret while the solution conductivity was monitored as a function of the volume of NaOH added with an Orion Benchtop Conductivity Meter (Model 162) equipped with an Orion Conductivity Cell (Model 013030) (Raymond, Morin & Marchessault, 1993). During the titration, the temperature of the solution was kept constant (30 °C) by using a water bath since the conductivity is a function of temperature. In a typical conductometric titration curve, there are two deflection points. The first deflection point corresponds to the neutralization of excess H⁺ ions of the strong acid, HCl. After all excess H⁺ ions are neutralized, then the neutralization of the weak acid, the ammonium salt in CS, starts. After the ammonium is completely neutralized, the conductivity again goes up with a higher value of slope due to the excess of OH⁻ ions of NaOH added, which is the second deflection point. Thus, the range between the first and the second deflection points corresponds to the neutralization of the protonated amine groups of CS. As a result, the number of moles of NaOH used between the first and second deflection points equals the number of moles of amine groups of the CS sample. The percent DD was calculated by the following equation:

$$\%DD = \frac{(v_2 - v_1) \times M_{NaOH} \left(\frac{mol}{L} \right) \times \frac{161.16g}{mol}}{Mass\ of\ chitosan\ sample\ (g)} \times 100 \quad (1)$$

where (v₂-v₁) is the difference in volume in liters between the two deflection points, M_{NaOH} is the molarity (mol/L) of standard NaOH solution, and 161.16 is the molar mass of chitosan.

2.5 Morphologic Characterization

CS particles were imaged via scanning electron microscopy (SEM) using a Phenom G1 (Model 800-03103-02) (FEI Co., Netherlands) microscope. Prior to imaging, a small amount of CS powder or flake was fixed on conductive carbon tape and mounted on the support. The sample was then sputtered with an approximately 6 nm layer of gold/palladium (Au/Pd) using a Quorum Technologies SC7620 Mini Sputter Coater (Laughton, East Sussex, UK), which deposits 10 nm coating/45 sec.

2.6 Hygroscopicity

Hygroscopicity of CS flake and powder was measured by loss on drying using a 120VAC Moisture Balance IL-50.001 (Summit Measurement Inc., South Deerfield, MA), an automatic moisture balance with a capacity of 50 g and resolution of 0.001 g. CS samples were placed on the balance and the masses before and after drying via vacuum were recorded. Loss on drying was calculated as the percent difference between the initial and final mass.

2.7 Molecular Weight

Viscosity of CS samples was measured using a 2–10 centistoke glass capillary ASTM 1 Ubbelohde viscometer (Fisher Scientific, Pittsburgh, PA). Viscosity measurements were performed by dissolving chitosan in a solvent containing 0.3 M acetic acid and 0.2 M sodium acetate (0.3 M AcOH/0.2 M NaOAc) to a final CS concentration of 1–2 mg/mL (0.1%–0.2%). The AcOH protonates the amino group which solubilizes CS and the NaOAc salt screens electrostatic interactions between different CS chains. The 0.3 M AcOH/0.2 M NaOAc solvent was chosen since previous studies have shown it prevents aggregate formation, unlike other solvents like 0.1 M AcOH/0.2 M NaCl (Rinaudo, Milas & Dung, 1993; Roberts & Domszy, 1982). Efflux times were measured for each of 3 concentrations (1 mg/mL, 1.5 mg/mL, and 2 mg/mL) and the following viscosities were determined using the given equations:

$$\text{Relative viscosity} \quad n_{\text{rel}} = \frac{t_{\text{CS}}}{t_{\text{sol}}} \quad (2)$$

$$\text{Specific Viscosity} \quad n_{\text{sp}} = n_{\text{rel}} - 1 \quad (3)$$

$$\text{Reduced viscosity} \quad n_{\text{red}} = \frac{n_{\text{sp}}}{c} \quad (4)$$

where t_{CS} represents efflux time for chitosan, t_{sol} represents efflux time for the solvent, and c represents chitosan concentration in g/mL. Intrinsic viscosity ($[\eta]$) was calculated using the Huggins plot in which the reduced viscosities for the 3 chitosan concentrations were extrapolated to infinite dilution (i.e. zero concentration). Intrinsic viscosity is related to molecular weight by the Mark-Houwink equation:

$$[\eta] = KM^{\alpha}$$

where K and a are constants for the specific polymer, solvent, and temperature combination. The K and a values for CS with a %DD of approximately 80% and the solvent 0.3 M AcOH/0.2 M NaOAc at 25 °C are 0.074 and 0.80, respectively (Brugnerotto, Desbrières, Roberts & Rinaudo, 2001).

2.8 FTIR

Molecular composition of CS flake and powder was assessed via Fourier Transform Infrared Spectroscopy (FTIR). To generate FTIR spectra, films of CS salts were cast by dissolving the CS flake or powder in 1% acetic acid. The films were then converted to the free amine form of CS by washing films in alcoholic base prior to imaging with a Nicolet iS50 FT-IR instrument (Thermo Fisher Scientific, Madison, WI) using a diamond attenuated total reflectance (ATR) attachment and 32 scans at 4 cm⁻¹ resolution.

2.9 Plasma Sterilization

CS samples were sterilized via NtNP treatment with either atmospheric pressure plasma (ApP) or low-pressure plasma (LpP) systems. The ApP instrument is a custom built instrument housed at North Carolina State University that has been described in detail previously and was operated at 4.8 kW power and 5 kHz frequency (Cornelius, 2007). Helium was used as the carrier gas at a flow rate of 10 L/min. The LpP instrument is the IoN40 plasma system (PVA-TePla America, Corona, CA) and the gas used was medical grade nitrogen, power was 500 Watts, pressure was maintained at 300 mTorr, and gas flow was 0.5 L/min.

Prior to NtNP treatment, CS samples were weighed and placed into paper-backed sterilization pouches. The pouches used in the ApP instrument were sealed and subsequently flushed with 100% nitrogen gas whereas the pouches used in the LpP instrument were left unsealed. The CS-containing pouches were placed into the ApP instrument or directly on the metal tray of the IoN40. For the LpP, the chamber was evacuated down to <100 mTorr and flushed with 100% nitrogen gas 3 times prior to beginning the NtNP treatment. Evacuating and flushing the chamber served to ensure the CS-containing pouches were filled completely with 100% N₂ gas instead of air.

Previous studies showed that sterility doses, defined as a 6-log reduction in bacterial spores, were achieved at 15 mins with both the ApP and LpP instruments when using nitrogen gas. As is commonly done in medical device and pharmaceutical manufacturing, an overkill dose of 2X the sterility dose, 30 minutes in this case, was used in these studies. Sterility of each NtNP-treated CS sample was confirmed by diluting each sample 40X with pyrogen-free water to overcome the anti-microbial properties of CS and subsequently cultured in standard polystyrene petri dishes containing tryptic soy agar at 37°C for 72 hours. Colony-forming units were enumerated by manually counting each culture plate.

2.10 Endotoxins

Endotoxins are measured in endotoxin units (EU), a unit of biological activity based on the United States Pharmacopoeia Reference Endotoxin Standard, since different types of endotoxins elicit varying degrees of immune responses. Endotoxins were extracted from CS

by incubating CS samples in a 40X volume of pyrogen-free water (w/v) for 2 hours at 37°C. The extracts were then syringe filtered through a 0.22 µm mixed cellulose esters filter (EMD Millipore, Inc., Darmstadt, Germany) to remove any residual CS that can cause false positives with Limulus amoebocyte lysate (LAL) assays. The endotoxin concentration in the filtrate was subsequently quantitated using a handheld LAL device (Endosafe PTS device, Charles River Laboratories, Charleston, South Carolina) and FDA-certified 0.5–0.005 EU/mL PTS cartridges. The LAL assay is the current gold standard method for quantitating endotoxins in drugs and medical devices.

3. RESULTS AND DISCUSSION

3.1 Particle Size Analysis

Cryomilling raw CS flake with either a cryo-ball or a cryo-jet mill resulted in a fine, white powder, as shown in Figure 1, consisting of mostly <100 µm particles. Average particle size and size distribution, including median and the diameter larger than 95% of the particles (D95), for each milling machine and parameter are presented in Table 1. Cryo-ball milling CS flakes for 15 minutes with 10 mm zirconium oxide balls generated CS powder with the largest mean particle size. Doubling the grind time to 30 minutes reduced the mean particle size by 21% and D95 by 15%. Using 50% smaller stainless steel balls reduced the mean particle size and D95 by an additional 57% and 55%, respectively. The smallest mean particle size generated by cryomilling (28.5 µm) was 24% smaller than the mean particle size generated by a standard ball mill after 8 hours (37.53 µm) in a previous study (Zhang, Zhang & Xia, 2014).

Cryo-jet milling CS for 30 minutes produced the smallest particles, with a mean particle size (16.05 µm) that was 44% smaller than the smallest mean particle size produced by cryo-ball milling (28.5 µm). Although the cryo-jet mill proved to be the superior method for milling CS into powder in the present study, a previous study showed that CS powder with a particle size distribution similar to cryo-jet milling can be produced with a cryo-ball mill when coupled with thermocatalytic destruction of CS (Laka & Chernyavskaya, 2006). Furthermore, another study found that CS flakes can be reduced to submicron sizes using an ultrafine milling technique (Zhang, Zhang, Jiang & Xia, 2012).

3.2 Viscosity and Molecular Weight

Cryomilling reduced the intrinsic viscosity and molecular weight (MW) of CS as shown in Figure 2. This reduction is a result of scissions of the chitosan polymer chain. A detailed discussion of this mechanism is provided in section 3.5. Since MW has a dramatic influence on the biological properties of CS, additional studies are needed to understand the influence of powders with different particle size distributions on biologic properties (Kumirska, Weinhold, Thöming & Stepnowski, 2011).

3.3 Degree of Deacetylation

Changes in the DD are shown in Figure 3. The 9.6% reduction in DD after cryo-jet milling appears to be another consequence of the scissions in the CS polymer chain that occur during cryomilling. Specifically, we postulate that chain breaks lead to changes in functional

groups in CS that may lead to chemical reactions. However, other mechanisms are possible. For example, high temperatures are known to occur, albeit for very short times ($< 10^{-6}$ seconds), at the fracture sites during cryo-jet milling and this might cause slight caramelization of the CS (Rumpf, 1973; Weichert & Schönert, 1976). Thus, additional studies are needed to better understand the mechanism(s) responsible for this change in %DD with cryomilling.

3.4 Hygroscopicity

The hygroscopicity of CS was increased by 25% after milling with loss on drying of the cryo-jet milled CS at 11.12% compared to 8.34% for the CS flake, reflecting the increase in surface area of the cryomilled material. The increase in hygroscopicity of CS after cryomilling is similar to the % loss on drying for the thermocatalytic ball milled CS powder reported by Laka and Chernyavskaya. The high hygroscopicity of CS powder must be taken into account when making CS solutions, which are commonly used for biomedical, and especially pharmaceutical, applications (Ahmadi, Oveisi, Samani & Amoozgar, 2015; Heffernan, Zaharoff, Fallon, Schlom & Greiner, 2011).

3.5 Morphologic Characterization

Morphologic characterization of the micronized CS via SEM imaging showed highly irregular particle shapes after cryo-jet milling, as shown in Figure 3. These irregular particle shapes are thought to be a result of the fact that most polymers at room temperature exhibit considerable tensile toughness or work to rupture, compared to glassy, brittle materials such as inorganic glasses. The appearance of the milled CS suggests the CS flakes are torn apart in a random fashion, leaving highly diverse particle shapes and sizes. The majority of particles appeared either sphere-like or spindle shaped. The size of these shapes is small enough that individual polymer chains are likely broken in the milling process, which leads to the reduced intrinsic viscosity and MW shown in Figure 2. For example, if we take 5.15×10^{-10} m as the diameter of the glucopyranose sugar ring, and assume that there is a degree of polymerization of 1000, then the CS chain is 0.5 μm in length and the particles range in diameter down to 16 μm and smaller.

3.6 FTIR

FTIR spectra for CS flake and powder are shown in Figure 4 and reveal changes in the molecular composition of CS after cryomilling. Specifically, the amine peak at 3400 cm^{-1} is lower in the CS powder compared to the CS flake, which correlates with the conductometric titration data and indicates loss of amine groups (is this the best way to state what the peak reduction indicates). There is no change in the amide I peak at 1640 cm^{-1} , but the CS powder shows a rightward shift at 1100 cm^{-1} compared to the CS flake. The significance of this peak shift is not currently understood.

3.7 Plasma Decontamination

Plasma treatment resulted in a sterile CS with endotoxin levels of 1.26 EU/g (ApP) and 2.46 EU/g (LpP). This is a very low level of endotoxins. Most medical or pharmaceutical grades of CS contain > 100 EU/g, although most are certified to be < 100 EU/g. Plasma sterilization

also resulted in a significant reduction in viscosity of both flake and powder forms of CS, as shown in Figure 6.

3.8 Solubility

In the process of making CS hydrogels for the tests described in previous sections, it was noted that cryomilled CS powder solubilized significantly faster and better in dilute acids than CS flakes. Thus, milling increased the solubility of CS. This is again a result of the increased surface area of the milled CS compared to the raw CS flake.

3.9 Biological Properties

Our lab has previously shown in a rat model of arterial and venous hemorrhage that the hemostatic properties of CS are preserved after NtNP-sterilization (Crofton, Chrisler, Hudson, Inceoglu, Petersen, & Kirsch, 2016). We have also found that micronized, NtNP-sterilized chitosan retains its drug delivery efficacy *in vivo* (unpublished data). Specifically, we treated mice bearing orthotopic bladder tumors with micronized, NtNP-sterilized CS and the anti-tumor cytokine IL-12 (CS+IL-12). We found there was no statistical difference in the anti-tumor efficacy of CS+IL-12 when the CS is micronized and NtNP-sterilized as described in the present study compared to its native form.

4. CONCLUSION

Cryo-ball and cryo-jet milling are effective methods for producing micron-sized CS particles and increasing the surface to volume ratio of CS. Cryo-milling also increases the hygroscopicity and decreases the %DD, intrinsic viscosity, and therefore the molecular weight, of CS. Cryo-jet milling produced a 44% smaller mean particle size than cryo-ball milling after 30 minutes of milling. However, in the case of cryo-ball milling, smaller grinding balls may produce much smaller mean particle sizes, potentially within the range of cryo-jet milling, but it is unclear whether there would be any advantage to cryo-ball milling over cryo-jet milling. Plasma-treated CS was sterile with <2.5 EU/g, but NtNP treatment caused a significant reduction in CS MW. This effect was greater with micronized CS powder, which is predicted to be due to the increased surface area of the powder. Future studies must be aimed at elucidating how different particle sizes influence biological properties of CS.

Acknowledgments

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under SBIR award number R43CA186374. This grant was awarded to Scion Cardio-Vascular, Inc. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors thank Jackie Knecht and Louis Rose for administrative assistance. The authors also thank Demetri Chrysostomou, Michael Barden, Luke Turalitsch, Eduardo Mateo, and James Bond for assistance with the IoN40 plasma instrument.

Abbreviations

CS	chitosan
NtNP	non-thermal nitrogen plasma

ApP	atmospheric pressure plasma
LpP	low-pressure plasma
DD	degree of deacetylation

References

- Ahmadi F, Oveisi Z, Samani SM, Amoozgar Z. Chitosan based hydrogels: characteristics and pharmaceutical applications. *Res Pharm Sci*. 2015; 10(1):1–16. [PubMed: 26430453]
- Artursson P, Lindmark T, Davis SS, Illum L. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharm Res*. 1994; 11(9):1358–1361. [PubMed: 7816770]
- Badawy MEI, Rabea EI. A Biopolymer Chitosan and Its Derivatives as Promising Antimicrobial Agents against Plant Pathogens and Their Applications in Crop Protection. *International Journal of Carbohydrate Chemistry*. 2011
- Brugnerotto J, Desbrières J, Roberts G, Rinaudo M. Characterization of chitosan by steric exclusion chromatography. *Polymer*. 2001; 42(25):09921–09927.
- Chien PJ, Li CM, Lee CH, Chen HH. Influence of Micronized Chitosan on Antioxidative Activities in Grape Juice. *Food and Nutrition Sciences*. 2013; 4(8):224–228.
- Cornelius, CE. PhD Dissertation. 2007. Atmospheric plasma characterization and mechanisms of substrate surface modification.
- Crofton AR, Chrisler J, Hudson SM, Inceoglu S, Petersen F, Kirsch WM. Effect of Plasma Sterilization on the Hemostatic Efficacy of a Chitosan Hemostatic Agent in a Rat Model. *Advances in Therapy*. 2016 E-pub ahead of print.
- de Oliveira Cardoso Macedo M, de Macedo HR, Gomes DL, de Freitas Daudt N, Rocha HA, Alves C Jr. Inhibition of microbial growth on chitosan membranes by plasma treatment. *Artif Organs*. 2013; 37(11):998–1002. [PubMed: 24251774]
- Dhital S, Shrestha AK, Flanagan BM, Hasjim J, Gidley MJ. Cryo-milling of starch granules leads to differential effects on molecular size and conformation. *Carbohydrate Polymers*. 2011; 84(3):1133–1140.
- Dodane V, Amin Khan M, Merwin JR. Effect of chitosan on epithelial permeability and structure. *Int J Pharm*. 1999; 182(1):21–32. [PubMed: 10332071]
- Dodane V, Vilivalam VD. Pharmaceutical applications of chitosan. *Pharmaceutical Science & Technology Today*. 1998; 1(6):246–253.
- Ehmer, A. PhD Dissertation. 2010. Micronization of proteins by jet milling.
- Franca R, Mbhe DA, Samani TD, Le Tien C, Mateescu MA, Yahia L, Sacher E. The effect of ethylene oxide sterilization on the surface chemistry and in vitro cytotoxicity of several kinds of chitosan. *J Biomed Mater Res B Appl Biomater*. 2013; 101(8):1444–1455. [PubMed: 24591223]
- Garnise R, Mar K, Crowder T, Hwang CR, Ferriter M, Huang J, Mikszta J, Sullivan V, Hickey A. Formulation of a dry powder influenza vaccine for nasal delivery. *AAPS PharmSciTech*. 2006; 7(1):131–137.
- Gimeno M, Ventosa N, Boumghar Y, Fournier J, Boucher I, Veciana J. Micronization of the chitosan derivatives d-Glucosamine Hydrochloride and d-Glucosamine Sulphate salts by dense gas anti-solvent precipitation techniques. *The Journal of Supercritical Fluids*. 2006; 38(1):94–102.
- Heffernan MJ, Zaharoff DA, Fallon JK, Schlom J, Greiner JW. In vivo efficacy of a chitosan/IL-12 adjuvant system for protein-based vaccines. *Biomaterials*. 2011; 32(3):926–932. [PubMed: 20965561]
- Jameela SR, Misra A, Jayakrishnan A. Cross-linked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs. *J Biomater Sci Polym Ed*. 1994; 6(7):621–632. [PubMed: 7873513]
- Koenings S, Sapin A, Blunk T, Menei P, Goepferich A. Towards controlled release of BDNF — Manufacturing strategies for protein-loaded lipid implants and biocompatibility evaluation in the brain. *Journal of Controlled Release*. 2007; 119(2):163–172. [PubMed: 17428570]

- Kristl J, Šmid-Korbar J, Štruc E, Schara M, Rupprecht H. Hydrocolloids and gels of chitosan as drug carriers. *International Journal of Pharmaceutics*. 1993; 99(1):13–19.
- Kumar M. A review of chitin and chitosan applications. *Reactive and Functional Polymers*. 2000; 46(1):1–27.
- Kumirska J, Weinhold MX, Thöming J, Stepnowski P. Biomedical activity of chitin/chitosan based materials—influence of physicochemical properties apart from molecular weight and degree of N-acetylation. *Polymers*. 2011; 3(4):1875–1901.
- Laka, M.; Chernyavskaya, S. *Proceedings-Estonian Academy of Sciences Chemistry*. Vol. 55. Truekitud Ou; 2006. Preparation of chitosan powder and investigation of its properties; p. 78
- Lim LY, Khor E, Koo O. Gamma irradiation of chitosan. *J Biomed Mater Res*. 1998; 43(3):282–290. [PubMed: 9730066]
- Lim LY, Khor E, Ling CE. Effects of dry heat and saturated steam on the physical properties of chitosan. *J Biomed Mater Res*. 1999; 48(2):111–116. [PubMed: 10331902]
- Luessen HL, de Leeuw BJ, Langemeyer MW, de Boer AB, Verhoef JC, Junginger HE. Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in vivo. *Pharm Res*. 1996; 13(11):1668–1672. [PubMed: 8956332]
- Marreco PR, da Luz Moreira P, Genari SC, Moraes AM. Effects of different sterilization methods on the morphology, mechanical properties, and cytotoxicity of chitosan membranes used as wound dressings. *J Biomed Mater Res B Appl Biomater*. 2004; 71(2):268–277. [PubMed: 15455369]
- Miranda A, Millán M, Caraballo I. Investigation of the influence of particle size on the excipient percolation thresholds of HPMC hydrophilic matrix tablets. *Journal of Pharmaceutical Sciences*. 2007; 96(10):2746–2756. [PubMed: 17506514]
- Mullarney MP, Leyva N. Modeling pharmaceutical powder-flow performance using particle-size distribution data. *Pharmaceutical Technology*. 2009; 33(3):126–134.
- Nordtveit RJ, Vårum KM, Smidsrød O. Degradation of fully water-soluble, partially N-acetylated chitosans with lysozyme. *Carbohydrate Polymers*. 1994; 23(4):253–260.
- Norzita Y, Norhashidah T, Maznah M, Nurul Aizam Idayu Mat S, Nor Akma S, Norafifah Ahmad F. Determination of viscosity-average molecular weight of chitosan using intrinsic viscosity measurement. *Journal of Nuclear and Related Technologies*. 2013; 10(1):39–44.
- Pillai CKS, Paul W, Sharma CP. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in Polymer Science*. 2009; 34(7):641–678.
- Rao SB, Sharma CP. Sterilization of chitosan: implications. *J Biomater Appl*. 1995; 10(2):136–143. [PubMed: 8618207]
- Raymond L, Morin FG, Marchessault RH. Degree of deacetylation of chitosan using conductometric titration and solid-state NMR. *Carbohydrate Research*. 1993; 246(1):331–336.
- Reverchon E, Antonacci A. Chitosan Microparticles Production by Supercritical Fluid Processing. *Industrial & Engineering Chemistry Research*. 2006; 45(16):5722–5728.
- Rinaudo M, Milas M, Dung PL. Characterization of chitosan. Influence of ionic strength and degree of acetylation on chain expansion. *International Journal of Biological Macromolecules*. 1993; 15(5): 281–285. [PubMed: 8251442]
- Roberts GAF, Domszy JG. Determination of the viscometric constants for chitosan. *International Journal of Biological Macromolecules*. 1982; 4(6):374–377.
- Rosiak J, Ula ski P, Kucharska M, Dutkiewicz J, Judkiewicz L. Radiation sterilization of chitosan sealant for vascular prostheses. *Journal of Radioanalytical and Nuclear Chemistry*. 1992; 159(1): 87–96.
- Rumpf H. Physical aspects of comminution and new formulation of a law of comminution. *Powder Technology*. 1973; 7(3):145–159.
- Saleem I, Smyth HC. Micronization of a Soft Material: Air-Jet and Micro-Ball Milling. *AAPS PharmSciTech*. 2010; 11(4):1642–1649. [PubMed: 21107775]
- San Juan A, Montebault A, Gillet D, Say JP, Rouif S, Bouet T, Royaud I, David L. Degradation of chitosan-based materials after different sterilization treatments. *IOP Conference Series: Materials Science and Engineering*. 2012; 31(1):012007.

- Schipper NM, Vårum K, Artursson P. Chitosans as Absorption Enhancers for Poorly Absorbable Drugs. 1: Influence of Molecular Weight and Degree of Acetylation on Drug Transport Across Human Intestinal Epithelial (Caco-2) Cells. *Pharm Res.* 1996; 13(11):1686–1692. [PubMed: 8956335]
- Takeuchi H, Yamamoto H, Niwa T, Hino T, Kawashima Y. Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-Coated Liposomes. *Pharm Res.* 1996; 13(6):896–901. [PubMed: 8792429]
- Tran TTB, Shelat KJ, Tang D, Li E, Gilbert RG, Hasjim J. Milling of Rice Grains. The Degradation on Three Structural Levels of Starch in Rice Flour Can Be Independently Controlled during Grinding. *Journal of Agricultural and Food Chemistry.* 2011; 59(8):3964–3973. [PubMed: 21384921]
- van der Lubben IM, Verhoef JC, van Aelst AC, Borchard G, Junginger HE. Chitosan microparticles for oral vaccination: preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches. *Biomaterials.* 2001; 22(7):687–694. [PubMed: 11246962]
- Weichert R, Schönert K. Temperatur at the point of fracture. *Chemie Ingenieur Technik.* 1976; 48(6): 543–544.
- Yao KD, Peng T, Yin YJ, Xu MX, Goosen MFA. Microcapsules/Microspheres Related to Chitosan. *Journal of Macromolecular Science, Part C.* 1995; 35(1):155–180.
- Zhang W, Zhang J, Jiang Q, Xia W. Physicochemical and structural characteristics of chitosan nanopowders prepared by ultrafine milling. *Carbohydrate Polymers.* 2012; 87(1):309–313.
- Zhang W, Zhang J, Xia W. Effect of Ball-Milling Treatment on Physicochemical and Structural Properties of Chitosan. *International Journal of Food Properties.* 2014; 17(1):26–37.

Highlights

- Chitosan was micronized by cryo-ball and cryo-jet milling.
- Mean particle size after cryo-jet mill (16.05 μm) was smaller than cryo-ball mill.
- Cryomilling reduced molecular weight by 26% and degree of deacetylation by 9.6%.
- Nitrogen plasma treatment produced sterile chitosan with endotoxins at <2.5 EU/g.
- Nitrogen plasma treatment also reduced molecular weight of chitosan.

Figure 1.
Macroscopic images of CS flake (left) and cryo-jet milled powder (right).

Figure 2. Effect of cryo-jet milling on intrinsic viscosity (A) and molecular weight (B) of CS. Flake is raw CS and powder is milled CS.

Figure 3.
Effect of cryo-jet milling on degree of deacetylation of CS. Flake is raw CS and powder is milled CS.

Figure 4. SEM images of CS flake before cryo-jet milling at 500X magnification (A) and resultant CS powder after cryo-jet milling at 1000X (B) and 5000X (C) magnification.

Figure 5. FTIR spectra of CS flake and powder showing changes in molecular composition caused by cryo-jet milling. Blue represents chitosan flake, red represents chitosan powder. Both flakes and powder were analyzed in the free amine form.

Figure 6. Changes in reduced viscosity of CS flake and cryo-milled powder after treatment with sterility and overkill doses of NtNP using LpP and ApP systems.

TABLE 1

Mean, Median and D95 values for cryomilled chitosan.

	Mean	Median	D95
Cryo-ball Mill (10 mm balls; 15 mins)	84.5 μm	71.0 μm	209.7 μm
Cryo-ball Mill (10 mm balls; 30 mins)	66.8 μm	52.2 μm	179.0 μm
Cryo-ball Mill (5 mm balls; 30 mins)	28.5 μm	20.4 μm	80.3 μm
Cryo-jet Mill (30 mins)	16.05 μm	15.62 μm	24.24 μm