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Chitosan powders and Ortho-R, a chitosan-containing finished product, are stable long term when stored in proper conditions

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ABSTRACT

Chitosan has sometimes been perceived to have a poor stability, which may explain why only a few chitosan-based products are available commercially. The purpose of this study was to investigate the stability of two chitosan powders and of Ortho-R (ChitogenX Inc), a chitosan-containing finished product that is used as an adjunct to improve repair of soft tissues. The powders and Ortho-R product were subjected to accelerated (40 °C/75 % RH) and long-term (25 °C/60 % RH) stability testing. The chitosan number average molar mass (M_n), polydispersity index (PDI), degree of deacetylation (DDA) and water content did not change substantially throughout the stability testing period and remained within the established specifications. Forced degradation studies were also performed on the Ortho-R product by exposing it to light, heat, humidity, hydrochloric acid and hydrogen peroxide. Storing the Ortho-R product in an open vial under warm and humid conditions (40 °C/75 % RH) led to an increase in chitosan M_n and PDI. The Ortho-R product was sensitive to oxidation as revealed by a decrease in chitosan M_n and PDI. Chitosan powders and the Ortho-R finished product are stable in the long term when the proper container closure systems are used.

1. Introduction

Although chitosan has been used extensively in research for decades (for example, 5837 chitosan-related publications were identified on Pubmed in 2023-2024), there are still only a few commercially available chitosan-based products (Yadav et al., 2019). Some authors have suggested that this is due to chitosan's poor stability and its susceptibility to processing conditions and/or environmental factors that could cause the polymer to degrade (Szymańska & Winnicka, 2015). Chitosan's intrinsic properties such as number average molar mass (M_n), degree of deacetylation (DDA), purity and water content affect its rate of degradation (Szymańska & Winnicka, 2015). It has been well established that chitosan is unstable in improper storage conditions. Exposing chitosan to high temperatures and light can change its molar mass and DDA (Dotto, Souza, & Pinto, 2011; Mucha & Pawlak, 2002; Wanjun, Cunxin, & Donghua, 2005). Exposure to low pH induces acid hydrolysis and a decrease of the polymer's molar mass (Laka & Chernyavskaya, 2006; Varum, Ottoy, & Smidsrod, 2001; Zoldners, Kiseleva, & Kaiminsh, 2005), a process that is temperature-dependent and can be slowed down by cold storage (Nguyen, Hein, Ng, & Stevens, 2008; No, Kim, Lee, Park, & Prinyawiwatkul, 2006). The US Pharmacopeia recommends that chitosan should be preserved in light-resistant and well-closed containers in a dry place, and stored at a temperature below 30°C (US Pharmacopeia. Chitosan monograph, 2019).

Ortho-R (ChitogenX Inc) is a chitosan-containing finished product that is used as an adjunct to improve surgical repair of soft tissues (Chevrier, Hurtig, & Lavertu, 2021; Dwivedi, Chevrier, Hoemann, & Buschmann, 2019; Ghazi zadeh et al., 2017). It is a freeze-dried powder of chitosan, trehalose and calcium chloride that is reconstituted in platelet-rich plasma (PRP), an autologous blood-derived component, prior to injection at the surgically repaired site, where it will solidify. The manufacturing process of Ortho-R involves three steps (Chevrier & Lavertu, 2024): 1) A starting chitosan material ($M_n \geq 150$ kDa and DDA ≤ 79 %) is deacetylated to a target 82 % DDA in sodium hydroxide in order to obtain the deacetylated chitosan intermediate CS82; 2) The deacetylated chitosan intermediate CS82 is depolymerized to a target M_n 42 kDa in nitrous acid in order to obtain the deacetylated and depolymerized chitosan CS8242; 3) The deacetylated and depolymerized chitosan CS8242 is solubilized in hydrochloric acid and mixed with trehalose (a lyoprotectant) and calcium chloride (an activator of

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PRP), and dispensed in individual vials for freeze-drying. The manufacturing processes for Ortho-R were developed in-house in our laboratory and then transferred to KABS Laboratories Inc, a contract manufacturing organization (CMO), that scaled up production and produced one engineering batch and then one good manufacturing practice (GMP)-compliant batch of the Ortho-R finished product.

The purpose of this study was to investigate the stability of chitosan powders (the deacetylated chitosan intermediate CS82 and the deacetylated and depolymerized chitosan CS8242) and of the Ortho-R finished product using a protocol that would follow ICH guidelines (Q1A R2) (ICH Guidelines, 2003). To our knowledge, accelerated and long-term stability testing of chitosan powders and a chitosan-containing product following ICH guidelines has not yet been described in the literature. We hypothesized that chitosan would be stable throughout the testing period, as long as proper container closure systems were used. More specifically, we hypothesized that chitosan M_n and DDA would not change substantially during accelerated (40 °C/75 % RH) and long-term (25 °C/60 % RH) stability testing.

2. Materials and methods

2.1. Preparation of the deacetylated chitosan intermediate CS82

ChitoClear® (Primex, Siglufjordur Iceland), a high purity grade chitosan isolated from Pandalus borealis chitin was used as starting material. Chitosan with *M*n 148 kDa, PDI 1.8 and 79.2 % DDA and chitosan with M_n 227 kDa, PDI 2.0 and 71.4 % DDA were used to produce the engineering and GMP-compliant batches respectively. Chitosans were placed in 5L reactors with NaOH (25 %, 6.25M) (Spectrum Chemical, New Brunswick, NJ, USA or Macron Fine Chemicals, Avantor, Haryana, India) and USP water (KABS Laboratories Inc, Saint-Hubert, QC, Canada) and deacetylated at 70 °C for 20 min (Eng batch) or 56 min (GMP batch). The deacetylated intermediate chitosans CS82 were rinsed with USP water (KABS Laboratories Inc, Saint-Hubert, QC, Canada), lyophilized and stored in double TWIRL'EM low-density polyethylene bags until depolymerization. The double layered bags were sealed with cable ties, then inserted into aluminum foil bags with five bags of Desi view (dessicant) in each foil bag. The foil bags were sealed individually with a heat sealer and inserted in a 3.5 gallon screw top pail with lid.

2.2. Preparation of the deacetylated and depolymerized chitosan CS8242

The deacetylated intermediate chitosans CS82 were dissolved in HCl (JT Baker, Avantor, Haryana, India) and USP water (KABS Laboratories Inc, Saint-Hubert, QC, Canada) in a 20L reactor and depolymerized with nitrous acid (Merck KGaA, Darmstadt, Germany) (pH < 2) at 50 °C for 3 h to produce the deacetylated and depolymerized chitosans CS8242. The conditions were the following for the depolymerization process: 0.5 % w/v chitosan in 50 mM HCl with 0.132 mM sodium nitrite (Eng batch) or 0.134 mM sodium nitrite (GMP batch). Insolubles were removed by filtration. The deacetylated and depolymerized chitosans were precipitated with NaOH (Spectrum Chemical, New Brunswick, NJ, USA or Macron Fine Chemicals, Avantor, Haryana, India), rinsed with USP water (KABS Laboratories Inc, Saint-Hubert, QC, Canada), sieved through a mesh and lyophilized. The deacetylated and depolymerized chitosans CS8242 were stored as described above (double polyethylene bags, foil bag and pail) until preparation of the Ortho-R finished product.

2.3. Preparation of the finished product Ortho-R (50 mg and 100 mg formats)

Two batches of the finished product Ortho-R were produced: 1) The engineering batch contained 50 mg of deacetylated and depolymerized chitosan CS8242 (M_n 37 kDa, PDI 1.7 and 81.7 % DDA) per vial, to be solubilized in 5 mL PRP for use and 2) The GMP batch contained 100 mg of deacetylated and depolymerized chitosan CS8242 (M_n 40 kDa, PDI 1.7 and 82.5 % DDA) per vial, to be solubilized in 10 mL PRP for use. The 50 mg format was used in pre-clinical studies (Chevrier, Hurtig, & Lavertu, 2021), which require less material, and the vial size was scaled up to 100 mg for clinical use. The deacetylated and depolymerized chitosans CS8242 were dissolved in HCl (JT Baker, JT Baker, Avantor, Haryana, India). The HCl concentration was adjusted so that 82 % (Eng batch) or 60 % (GMP batch) of the chitosan amino groups were protonated. The HCl concentration used will impact clotting kinetics upon solubilization with PRP; Finished product prepared at lower protonation levels solidify more quickly and yield clots that are stiffer (Chevrier & Lavertu, 2024). The dissolved deacetylated and depolymerized chitosans CS8242 were mixed with calcium chloride (Spectrum Chemical, New Brunswick, NJ, USA, final concentration 42 mM, to allow for solidification of PRP) and trehalose (Pfansthiel, Waukegan, IL, USA, final concentration 1 % w/v, a lyoprotectant for freeze-drying). The solutions were filtered through a 0.2 micron membrane filter and dispensed into individual glass vials (5 mL into 10 mL vials for the Eng batch and 10 mL into 20 mL vials for the GMP-compliant batch). The freeze-drying cycle was the following: 1) Ramped freezing to -5 °C in 200 min, 2) -5 °C for 1200 min, 3) Ramped heating to 5 °C in 60 min, 4) 5 °C for 840 min, 5) Ramped heating to 20 °C in 60 min, 6) 20 °C for 840 min, all at 120 millitorr. The container closure system for the finished product Ortho-R consisted of Type I borosilicate vials, chlorobutyl stoppers and flip off seals.

2.4. Stability testing program

Accelerated stability testing was performed in a stability chamber at 40°C/75 % RH and long-term stability testing was performed at 25 °C/60 % RH. Only GMP-compliant produced deacetylated chitosan intermediate CS82 and deacetylated and depolymerized chitosan CS8242 were tested, while both batches of finished product Ortho-R (50 mg and 100 mg) were tested. For the deacetylated chitosan intermediate CS82 and the deacetylated and depolymerized chitosan CS8242, individual batches of 0.1 g or 0.5 g of chitosan were stored in the container closure system described above (double polyethylene bags, foil bag and pail) and retrieved for testing. For the finished product Ortho-R (50 mg and 100 mg), vials were stored upright and in inverted positions and only the inverted vials were tested. Testing was performed at 0, 3 and 6 months for the accelerated program (CS82, CS8242, Ortho-R 50 mg, Ortho-R 100 mg). Testing was performed at 0, 3, 6, 9, 12, 18 months (CS82, CS8242, Ortho-R 50 mg, Ortho-R 100 mg) and at 24 and 36 months (Ortho-R 50 mg) for the long-term program.

2.5. Forced degradation studies

In the context of qualifying the size exclusion chromatography-small angle light scattering (SEC-MALS) method described below, the possible routes of degradation of Ortho-R were evaluated in forced degradation studies. For photolysis, finished product Ortho-R (50 mg format) was transferred into a quartz bottle. The bottle was placed in a photo stability study chamber and simultaneously exposed to both cool white fluorescent (whole spectrum, 10 500 Lux) and ultraviolet light (100-400 nm, 1.00 mW/cm^2) for 7 days. For thermal degradation, finished product Ortho-R (50 mg) was stored at 60 °C (in an oven) or 80 °C (in an oven) for 7 days. It was also stored in a stability chamber at 40 °C/75 % RH, with caps removed (open) or not (closed) for 7 days. All thermal degradation samples were protected from light. For acid degradation, finished product Ortho-R was reconstituted in 0.1N HCl and stored at room temperature for 7 days, protected from light. For oxidative degradation, finished product Ortho-R was reconstituted in 10 % (v/v) H_2O_2 and stored at room temperature for 7 days, protected from light.

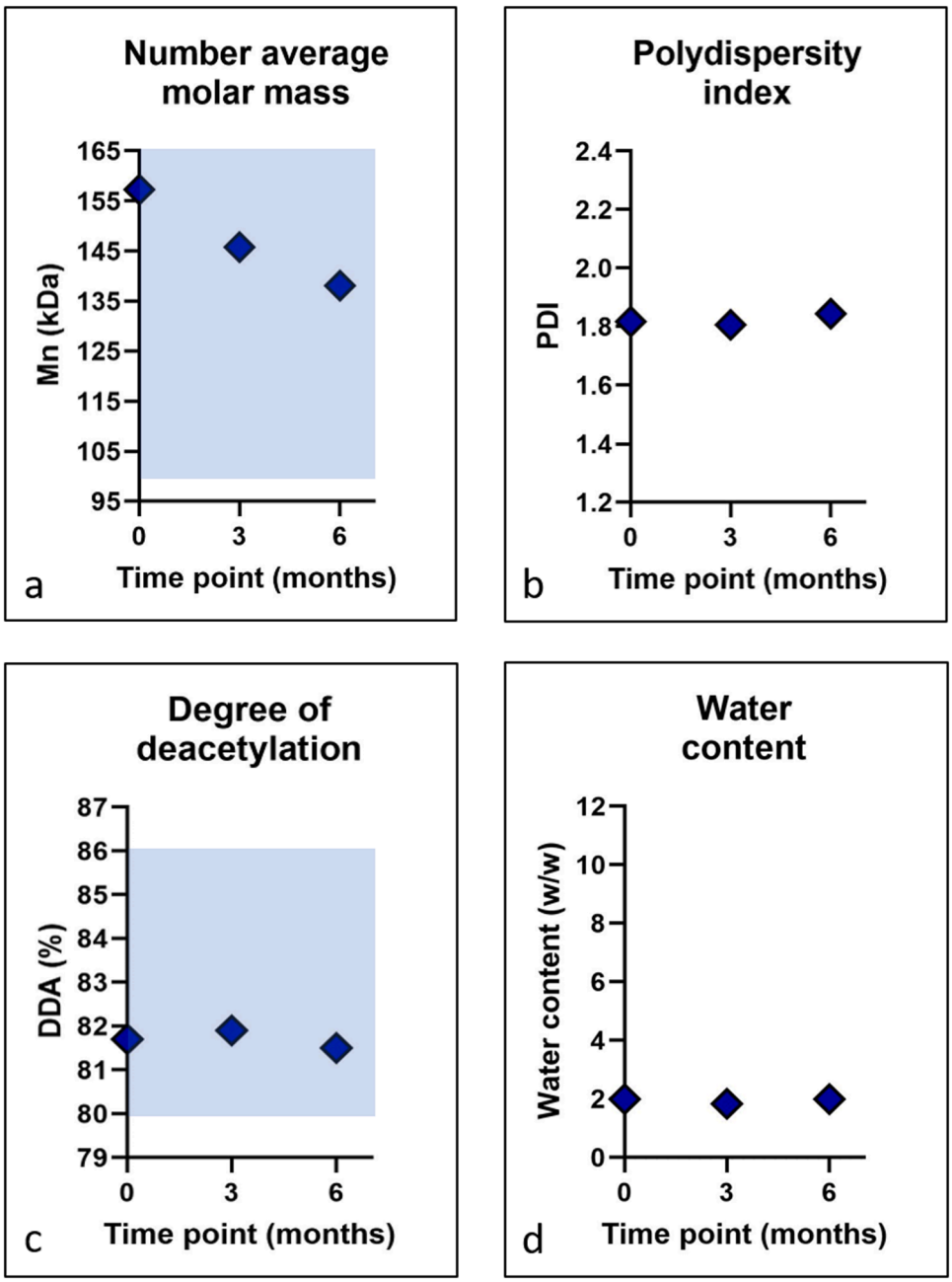

Fig. 1. Number average molar mass (a), polydispersity index (b), degree of deacetylation (c) and water content (d) of the deacetylated chitosan intermediate CS82 subjected to accelerated stability testing at 40 °C/75 % RH. Results are shown as average ± SD. Note that the error bars are smaller than the symbols. n = 2-4 replicates for each except degree of deacetylation n = 1. Specifications at shelf life are shaded in blue. The specifications for polydispersity index and water content were to report the measured values.

2.6. Identification testing

The Fourier transform infrared (FT-IR) spectrum was obtained using USP method < l97K> (US Pharmacopeia, 2025c) which allows the acquisition of very well defined spectrum with a strong band in a region 3422 - 522 cm-1. (Supplementary Figure S1). The structure of chitosan was confirmed by ^{1}H NMR (Lavertu et al., 2003) (Supplementary Figure S2). Identification was based on the presence of the following signals: 1) a peak corresponding to the 3 acetyl protons must be present around 2.3 ppm which may be small in the case of a high DDA; 2) the H2D peak must be observed around 3.5 ppm; 3) signals corresponding to H2A, H3, H4, H5, and H6 must be present in the region between 3.6 and 4.2 ppm (in the case of Ortho-R finished product, may be obscured by the trehalose excipient peaks); and 4) the H1D peak must be observed around 5.2 ppm. Identification and ^{1}H NMR testing was outsourced to Element (9240 Santa Fe Springs Rd, Santa Fe Springs, CA, USA 90670).

2.7. Determination of molar mass, polydispersity index and degree of deacetylation

Chitosan M_n and PDI were determined by SEC-MALS (Nguyen, Winnik, & Buschmann, 2009) using a Shimadzu Prominence-i 2030C Model LC2030C Plus HPLC equipped with a MALS detector Wyatt miniDAWN TREOS II Model WTS2-02 and a differential refractometer (dRI) detector Wyatt Optilab TrEX Model WTREX-13. SEC-MALS works by simultaneously measuring the concentration and light scattering

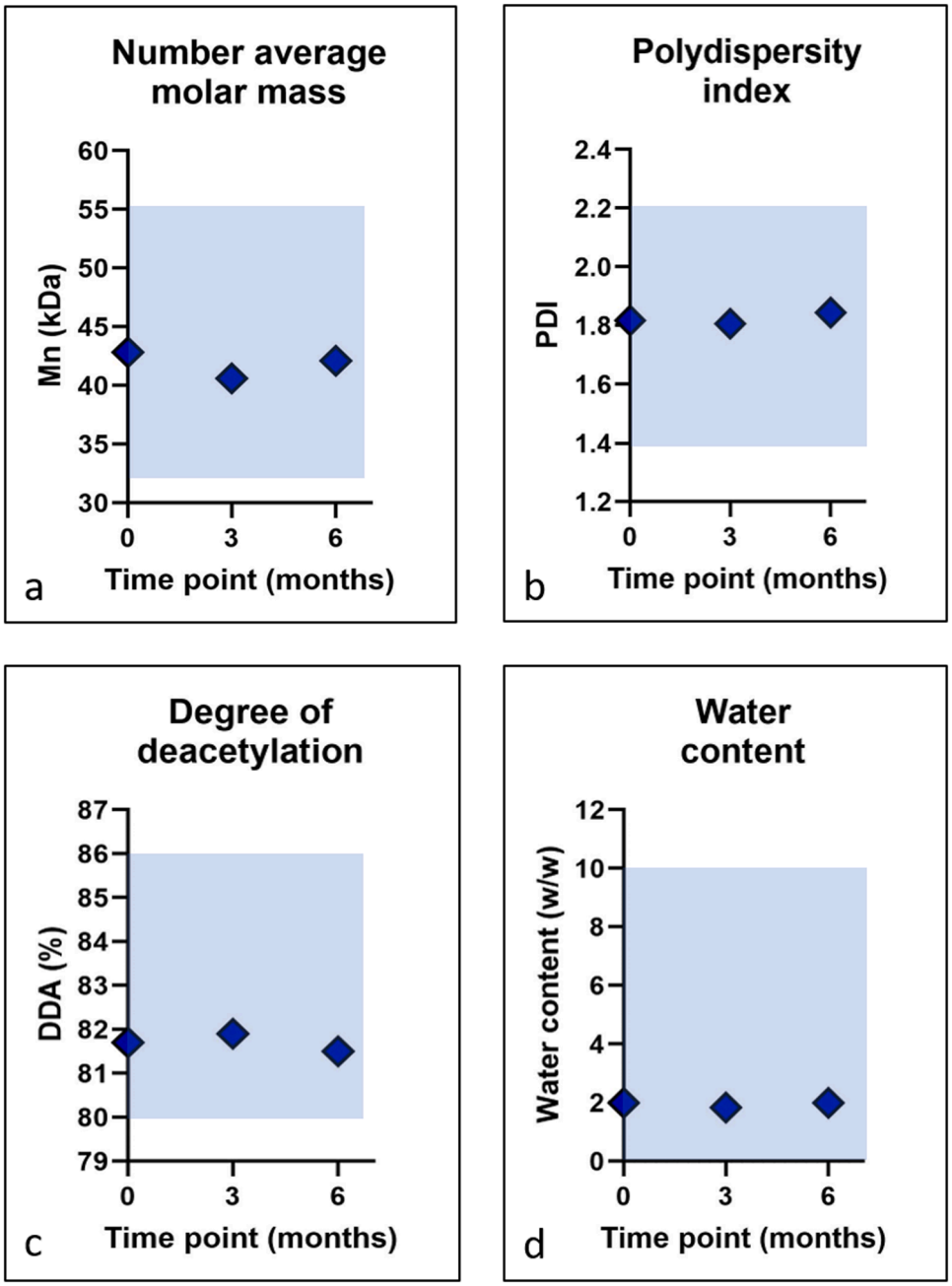

Fig. 2. Number average molar mass (a), polydispersity indes (b), degree of deacetylation (c) and water content (d) of the deacetylated and depolymerized chitosan CS8242 subjected to accelerated stability testing at 40 °C/75 % RH. Results are shown as average ± SD. Note that the error bars are smaller than the symbols. n = 2-3 replicates for each except degree of deacetylation n = 1. Specifications at shelf life are shaded in blue.

intensity of a sample solution with a previously determined dn/dc value, to determine the molar mass of the sample. By coupling this measurement to the size-based separation offered by SEC, the molar mass distribution (and thus the polydispersity) of the sample can also be determined. All samples were diluted in 0.1M sodium acetate/0.4 mM sodium azide pH 4.5 for testing. The HPLC system equipped with the dRI detector was used for dn/dc determination. The HPLC system equipped with the dRI and the MALS detector was used for molar mass determination. The latter was equipped with a guard column (TSK Gel PWXL-CP 13 μm), and two columns (TSK Gel G6000PWXL-CP 13 μm and TSK Gel G5000PWXL-CP 10 μm). An example of a SEC-MALS tracing can be found in supplementary figures (Supplementary Figure S3). The degree of deacetylation (%DDA) of chitosan was determined by 1H NMR spectroscopy (Lavertu et al., 2003) by Element (9240 Santa Fe Springs Rd, Santa Fe Springs, CA, USA 90670). The DDA was calculated using integrals of the peak of proton H1 of deacetylated monomer (H1-D) and of the peak of the three protons of acetyl group (H-Ac): $\left(\frac{H1D}{H1D+\frac{HAc}{3}}\right) \times 100$.

2.8. Determination of water content, chitosan content and calcium chloride in each vial

Water content was measured as per USP <921>Ic (US Pharmacopeia, 2025e). The SEC-MALS analysis technique described above was used to determine chitosan content in each vial, using the signal from the refractive index detector. A reference chitosan material (the

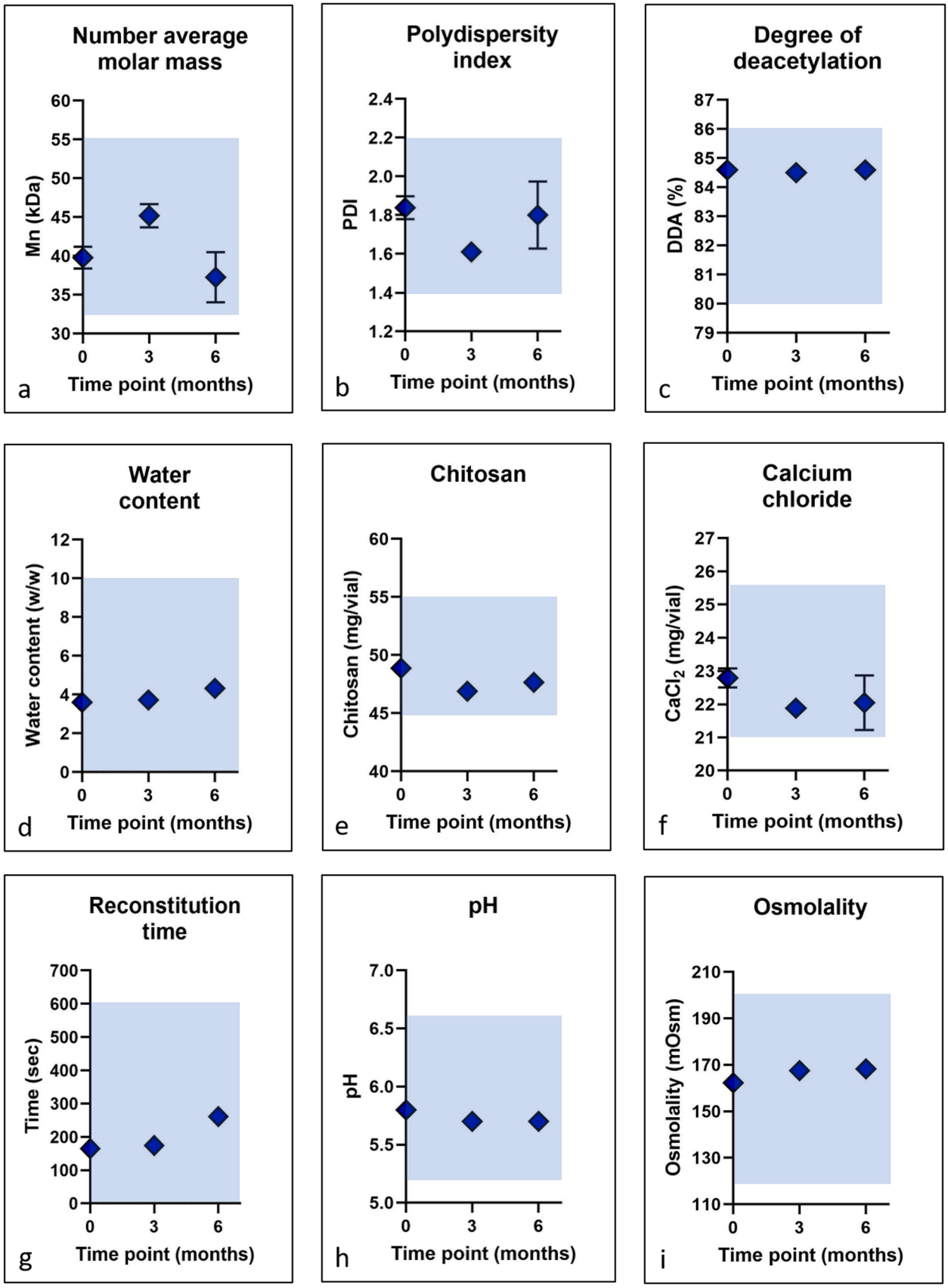

Fig. 3. Number average molar mass (a), polydispersity index (b), degree of deacetylation (c), water content (d), chitosan content (e) and $CaCl_2$ content (f) of the finished product Ortho-R (50 mg format) subjected to accelerated stability testing at 40 °C/75 % RH. Reconstitution time in water (g) and pH (h) and osmolality (i) of the resulting solution are also shown. Results are shown as average ± SD. Note that the error bars are smaller than the symbols in some cases. n = 2-8 replicates for each except degree of deacetylation, reconstitution time and pH n = 1. Specifications at shelf life are shaded in blue.

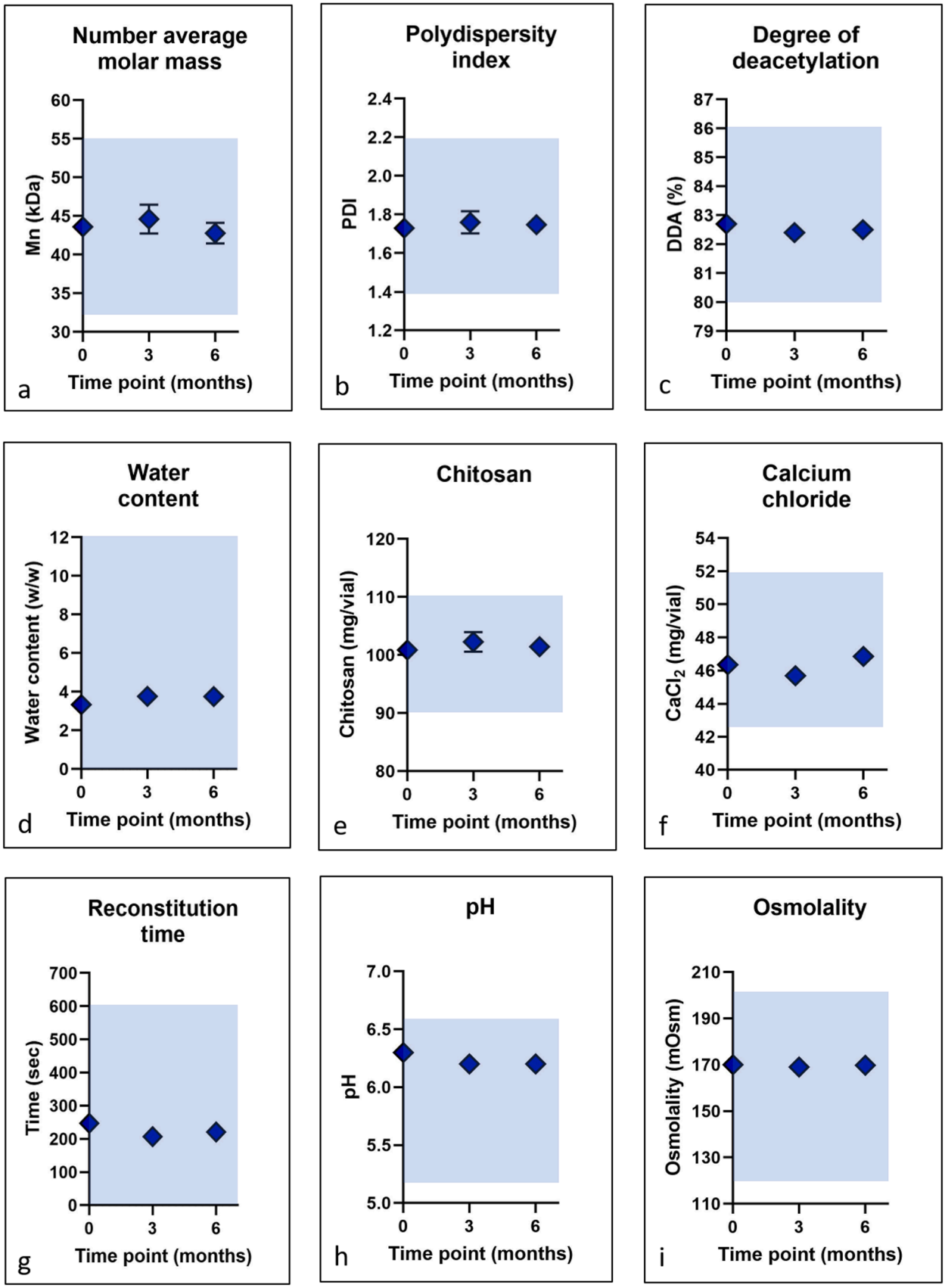

Fig. 4. Number average molar mass (a), Polydispersity index (b), degree of deacetylation (c), water content (d), chitosan content (e) and $CaCl_2$ content (f) of the finished product Ortho-R (100 mg format) subjected to accelerated stability testing at 40 °C/75 % RH. Reconstitution time in water (g) and pH (h) and osmolality (i) of the resulting solution are also shown. Results are shown as average ± SD. Note that the error bars are smaller than the symbols in some cases. n = 2-6 replicates for each except degree of deacetylation, reconstitution time and pH n = 1. Specifications at shelf life are shaded in blue.

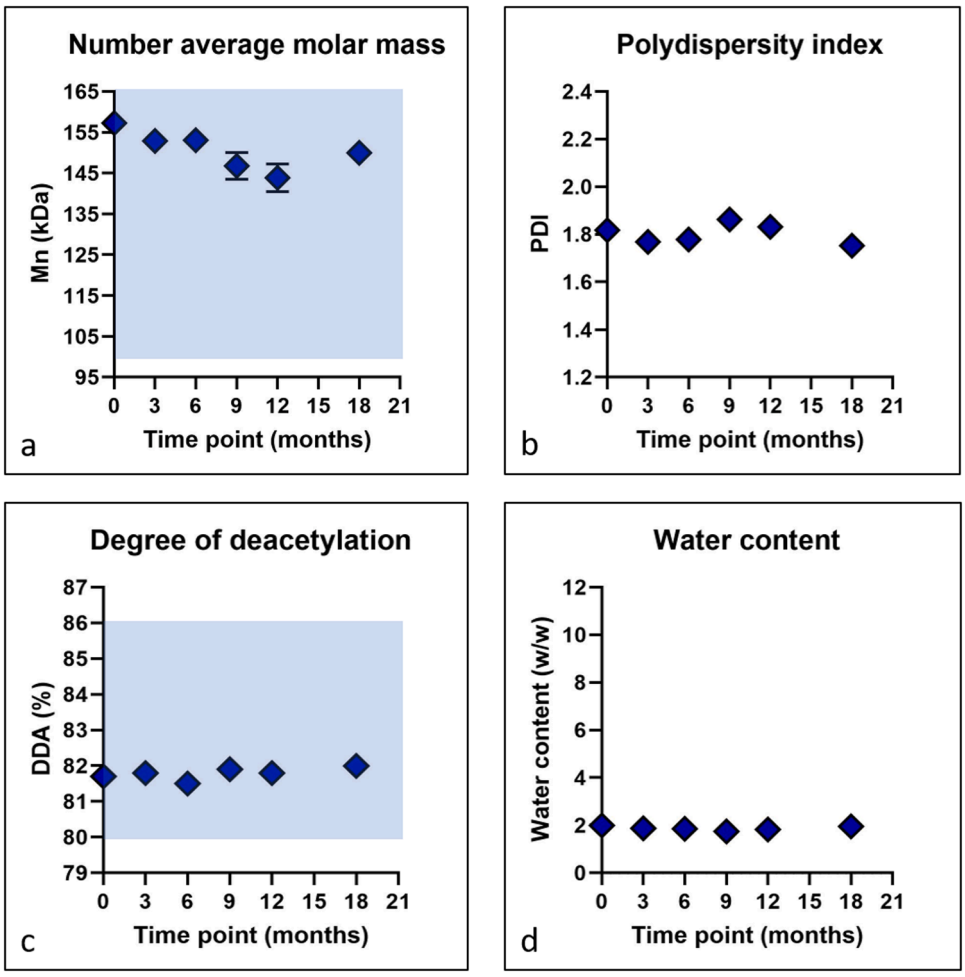

Fig. 5. Number average molar mass (a), polydispersity index (b) and degree of deacetylation (c) of the deacetylated chitosan intermediate CS82 subjected to long-term stability testing at 25 °C/60 % RH. Results are shown as average ± SD. Note that the error bars are smaller than the symbols in some cases. n = 2-4 replicates for each except degree of deacetylation n = 1. Specifications at shelf life are shaded in blue.

deacetylated and depolymerized CS8242) was run on the system prior to running the samples. The RI peak areas of the reference materials and of the samples were compared to calculate the assay value of the Ortho-R finished product. $CaCl_2$ content in the Ortho-R finished product was measured by complexometric titration.

2.9. Reconstitution in water and additional testing

Finished product Ortho-R was reconstituted in water for injection (5 mL water for the 50 mg format and 10 mL water for the 100 mg format). pH and osmolality were measured. USP <788> (US Pharmacopeia, 2025d) methods were used to quantify particulate matter in the reconstituted solutions.

2.10. Reconstitution in platelet-rich plasma and additional testing

Commercial citrated sheep blood (Cedarlane, Burlington, ON, Canada) was double centrifuged at 800 g for 10 min then 600 g for 20 min in order to isolate PRP. Finished product Ortho-R was reconstituted in PRP (5 mL PRP for the 50 mg format and 10 mL PRP for the 100 mg format) and shaken vigorously for 20 s. 250 µL of the mixture was then added to 2 pre-heated glass tubes and left for 30 min on a heat block (37 °C). After 30 min, the formulation was verified for clotting by lifting and tilting the glass tubes. The resulting clots were fixed in 10 % neutral buffered formalin, paraffin-embedded, sectioned at 5 µm thickness and stained with Weigert Iron Hematoxylin and Cibacron Brilliant Red (Rossomacha, Hoemann, & Shive, 2004) (Supplementary Figure S4). Chitosan dispersion in the clots was then scored qualitatively for homogeneity, with a score of +, ±, and -.

2.11. Sterility testing and endotoxin quantification

Sterility testing was outsourced to NucroTechnics (2000 Ellesmere Rd Scarborough, ON, Canada M1H 2W4) and performed as per USP <71> (US Pharmacopeia, 2025a). Endotoxin testing was outsourced to Associates of Cape Cod (124 Bernard St Jean Dr, East Falmouth, MA, USA 02536) and followed USP <85> (US Pharmacopeia, 2025b).

3. Results

3.1. Accelerated stability testing

Accelerated stability testing was performed at 40 °C/75 % RH for 6 months as recommended in the ICH guidelines (Q1A R2) (ICH Guidelines, 2003). All data were within specifications at all time points tested for the deacetylated chitosan intermediate CS82 (Fig. 1), the deacetylated and depolymerized chitosan CS8242 (Fig. 2) and the Ortho-R finished product 50 mg (Fig. 3) and 100 mg (Fig. 4) formats. Of note, the chitosan number average molar mass (M_n), polydispersity index (PDI), degree of deacetylation (DDA) and water content did not change substantially throughout the testing period (Figs. 1 to 4).

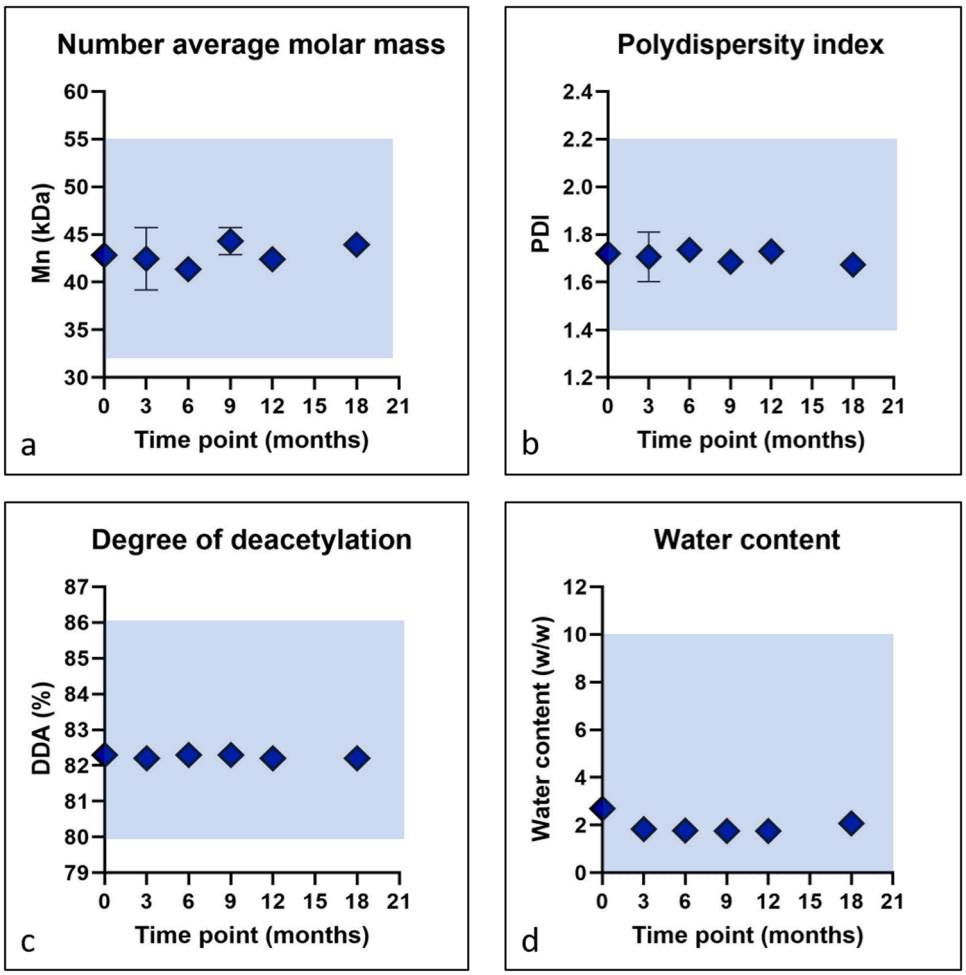

Fig. 6. Number average molar mass (a), polydispersity index (b), D degree of deacetylation (c) and water content (d) of the deacetylated and depolymerized chitosan CS8242 subjected to long-term stability testing at 25 °C/60 % RH. Results are shown as average ± SD. Note that the error bars are smaller than the symbols in some cases. n = 2-4 replicates for each except degree of deacetylation n = 1. Specifications at shelf life are shaded in blue.

3.2. Long-term stability testing

Long-term stability testing was performed at 25 °C/60 % RH as recommended the ICH guidelines (Q1A R2). Testing was until 18 months for the deacetylated chitosan intermediate CS82 (Fig. 5), the deacetylated and depolymerized chitosan CS8242 (Fig. 6) and the Ortho-R finished product 100 mg format (Fig. 8), while the Ortho-R finished product 50 mg format was tested until 36 months (Fig. 7). All data were within specifications at all time points tested. Importantly, there were no substantial changes to chitosan number average molar mass (M_n), polydispersity index (PDI), degree of deacetylation (DDA) and water content (Figs. 5 to 8).

3.3. Additional testing

IR spectra corresponded to the reference standard (Supplementary Figure S1 and Supplementary Tables S1 to S4). NMR spectra were consistent with chitosan structure and had the characteristic peaks (Supplementary Figure S2 and Tables S1 to S4). Endotoxin content was 8.7 EU/g for the Ortho-R finished product 50 mg format and < 3.85 EU/g for the 100 mg format (Supplementary Tables S1 to S4), well below the ≤ 100 EU/g specification. Ortho-R finished product vials remained sterile until the end of the stability testing programs, both in accelerated conditions (until 6 months) and in long-term conditions (until 36 months for the 50 mg format and until 18 months for the 100 mg format) (Supplementary Tables S1 to S4). Particulate matter was well below the established specifications for both Ortho-R finished product formats (Supplementary Tables S1 to S4). Finally, the formulations coagulated upon reconstitution in PRP and dispersion of the deacetylated and depolymerized chitosan CS8242 was homogenous in the resulting clots (Supplementary Figure S4 and Supplementary Tables S1 to S4).

3.4. Forced degradation studies

Finished product Ortho-R (50 mg) was not very sensitive to heat or light, but was sensitive to high humidity, as shown by substantial changes in M_n and PDI (Table 1). Storing the product in an open vial in warm, humid conditions (40 °C/75 % RH) caused substantial increases to both after only one week of storage (Table 1). The open sample also changed color and became darker (going from a cream/white color to an off-white beige color). The Ortho-R sample stored sealed under the same conditions was stable after one week (Table 1). The Ortho-R sample that was stored for 7 days in a low pH environment was also stable (Table 1). The Ortho-R sample was however sensitive to degradation by oxidation, as shown by a strong decrease in M_n along with a smaller decrease in PDI (Table 1).

4. Discussion

The data supported our starting hypothesis. The chitosan powders (the deacetylated chitosan intermediate CS82 and the deacetylated and depolymerized chitosan CS8242) as well as the Ortho-R finished product

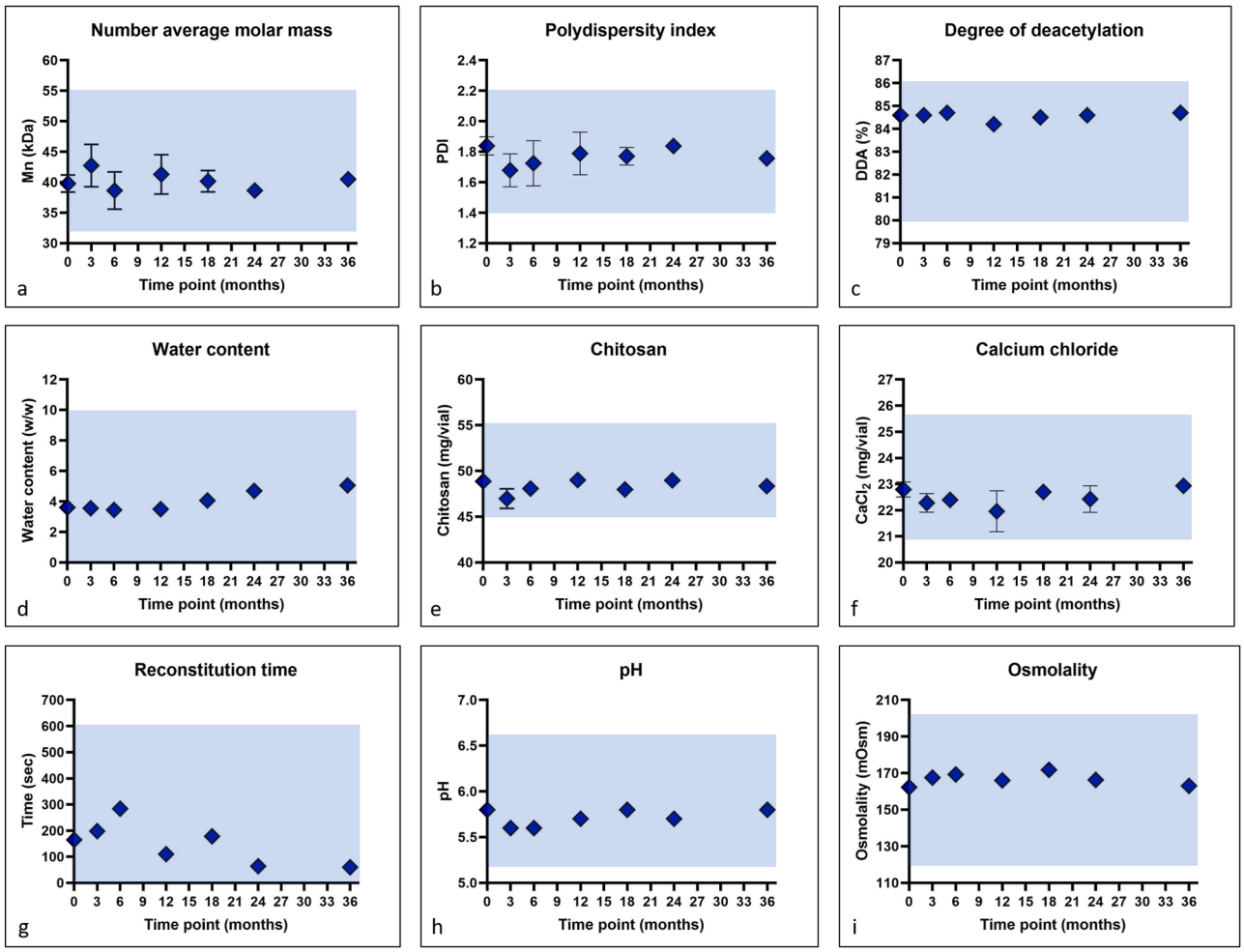

Fig. 7. Number average molar mass (a), polydispersity index (b), degree of deacetylation (c), water content (d), chitosan content (e) and $CaCl_2$ content (f) of the finished product Ortho-R (50 mg format) subjected to long-tern stability testing at 25 °C/60 % RH. Reconstitution time in water (g) and pH (h) and osmolality (i) of the resulting solution are also shown. Results are shown as average ± SD. Note that the error bars are smaller than the symbols in some cases. n = 2-8 replicates for each except degree of deacetylation, reconstitution time and pH n = 1. Specifications at shelf life are shaded in blue.

(50 mg and 100 mg formats) were found to be stable when stored in proper container closure systems. Of particular importance, the M_n and DDA of the deacetylated and depolymerized chitosan CS8242 as well as the Ortho-R finished product (50 mg and 100 mg formats) did not substantially change in either accelerated or long-term storage conditions and remained within established specifications (32-55 kDa for M_n and 80-86 % for DDA). Both parameters were previously found to be critical for Ortho-R finished product performance when reconstituted with PRP (Chevrier et al., 2018; Deprés-Tremblay, Chevrier, Tran-Khanh, Nelea, & Buschmann, 2017). Chitosans with Mn 32-55 kDa are soluble in PRP and the resulting clots are homogenous, with chitosan well dispersed throughout the clots. Higher M_n chitosans are not soluble in PRP and lower M_n chitosans lead to phase separation of the polymer and the blood components. Higher DDA chitosans induce instant agglutination of the red blood cells that are present in platelet-rich plasma which compromises handling and injectability. As the Ortho-R finished product is intended to be injected in the body to stimulate soft tissue repair, we elected to set the lower DDA specification to 80 % based on previous studies showing that lower DDA chitosans are degraded rapidly and elicit significant inflammatory reactions in vivo (Hidaka, Ito, Mori, Yagasaki, & Kafrawy, 1999; Tomihata & Ikada, 1997).

The forced degradation studies were performed in order to validate the suitability of the SEC-MALS analytical method to detect potential degradation products that could have been generated during the course of the stability studies. Although chitosan has been found to be sensitive to degradation by photolysis and elevated temperatures previously (Dotto et al., 2011; Mucha & Pawlak, 2002; Szymańska & Winnicka, 2015; Wanjun et al., 2005), here, the Ortho-R finished product samples that were stored sealed under light or high temperatures were all consistent with the unstressed sample after one week. Only in the sample left open at 40 °C/75 % RH did the M_n and PDI increase. The color of the sample had also changed, one of the most basic indications of a chemical reaction. These changes indicate that the deacetylated and depolymerized chitosan CS8242 in the Ortho-R finished product began to cross-link after being stored open at 40 °C/75 % RH for one week. The cross-linking is most probably attributable to a Maillard reaction occurring in which the aldehyde of the open form of the chain terminal unit reacts with the amino groups of another chain to form a longer chain through an imine function (Tommeraas, Varum, Christensen, & Smidsrod, 2001). Choosing the appropriate container closure system and ensuring that vials are well sealed will contribute to the product's stability. Most importantly, preventing water from entering the container closure systems is critical, as moisture content is one factor that plays a crucial role in determining the mechanism and the speed of chitosan degradation (Szymańska & Winnicka, 2015). Of note, water content was way below the established specification (<10 % w/w) throughout the accelerated and long-term stability studies for the deacetylated chitosan intermediate CS82, the deacetylated and depolymerized chitosan CS8242 and the Ortho-R finished product,

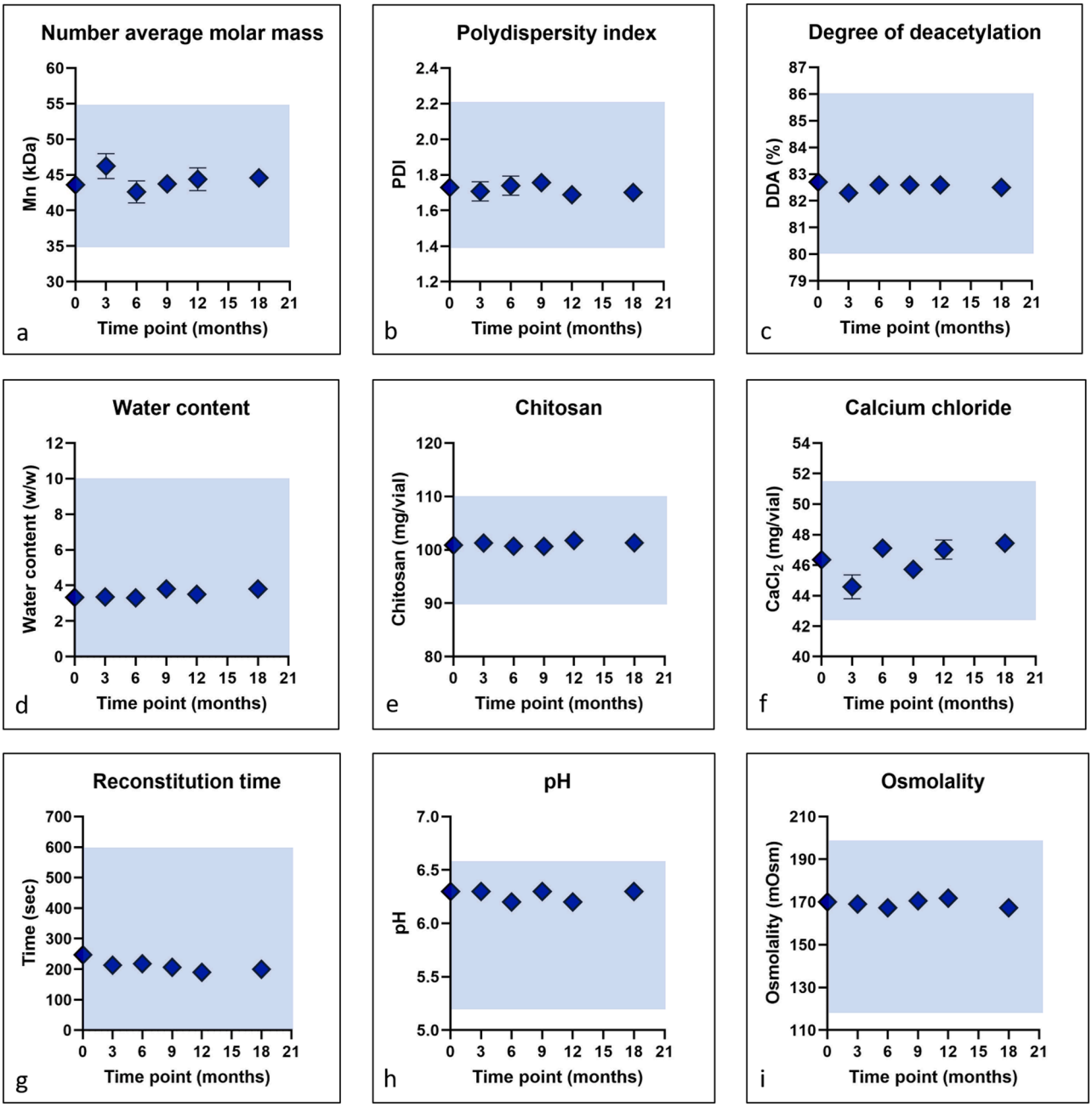

Fig. 8. Number average molar mass (a), polydispersity index (b), degree of deacetylation (c), water content (d), chitosan content (e) and $CaCl_2$ content (f) of the finished product Ortho-R (100 mg format) subjected to long-term stability testing at 25 °C/60 % RH. Reconstitution time in water (g) and pH (h) and osmolality (i) of the resulting solution are also shown. Results are shown as average ± SD. Note that the error bars are smaller than the symbols in some cases. n = 2-6 replicates for each except degree of deacetylation, reconstitution time and pH n = 1. Specifications at shelf life are shaded in blue.

suggesting that storage conditions were appropriate in this case.

The Ortho-R finished product was not very sensitive to the effects of a low pH environment contrary to what has been reported in the literature (Laka & Chernyavskaya, 2006; Nguyen, Hein, Ng, & Stevens, 2008; No, Kim, Lee, Park, & Prinyawiwatkul, 2006; Varum, Ottoy, & Smidsrod, 2001; Zoldners, Kiseleva, & Kaiminsh, 2005). This is probably related to the vial's storage conditions (room temperature instead of under heat) and the relatively short time of exposure (7 days). Dilute acidic conditions similar to ours (0.1 M HCl) have been shown to cause hydrolysis of chitosan from 10-103h (Varum, Ottoy, & Smidsrod, 2001), but testing in that study was at 83 °C, and not at room temperature. The deacetylated and depolymerized chitosan CS8242 in the Ortho-R finished product was sensitive to degradation by oxidation; the number average molar mass had significantly decreased after exposure to H_2O_2 for 7 days. This indicates that the hydrogen peroxide had degraded the product by chain scission. In addition to the molar mass of the deacetylated and depolymerized chitosan CS8242 decreasing, the polydispersity of the sample had also decreased. The most likely hypothesis to explain this change is that the chain scission process is not random, but is in fact preferential towards the longer polymer chains. A depolymerization by a random chain scission process should result in a polydispersity of 2, independent of the PDI of the starting polymer (Yoon, Chin, Kim, & Kim, 1996). Given that the polydispersity of the sample had decreased from a value of 1.8 for the unstressed sample to a value of 1.4 for the sample degraded by hydrogen peroxide, it seems that the degradation process may be preferential for the longer polymer chains.

Table 1
Number average molar mass (M_n) and polydispersity index (PDI) of the Ortho-R finished product (50 mg format) subjected to forced degradation testing. n = 1 for each condition.

Condition	M_n (kDa)	PDI
Unstressed	41	1.8
Photolysis RT (7days)	39	1.8
80 °C (7 days)	38	1.9
60 °C (7 days)	41	1.8
40 °C/75 % RH (7 days, closed)	38	1.9
40 °C/75 % RH (7 days, open)	54	2.4
0.1N HCl RT (7 days)	39	1.8
10 % H_2O_2 RT (7 days)	6	1.4

RT: Room temperature.

In summary, we have shown here that chitosan powders (the deacetylated chitosan intermediate CS82 and the deacetylated and depolymerized chitosan CS8242) and the Ortho-R finished product (50 mg and 100 mg formats) can be stored for long periods of time without undergoing significant changes, as long as the proper container closure systems are used.

Figure S1. FT-IR spectrum of chitosan.

Figure S2. 1H NMR spectrum of chitosan.

Figure S3. SEC-MALS tracing of a deacetylated chitosan intermediate (CS82).

Figure S4. Cibacron Brilliant Red-stained paraffin section of a clot obtained by reconstituting the finished product Ortho-R in platelet-rich plasma.

Table S1. Additional testing performed on the finished Ortho-R product (50 mg format)-Accelerated conditions (40 °C/75 % RH).

Table S2. Additional testing performed on the finished Ortho-R product (50 mg format)-Long-term conditions (25 °C/60 % RH).

Table S3. Additional testing performed on the finished Ortho-R product (100 mg format)-Accelerated conditions (40 °C/75 % RH).

Table S4. Additional testing performed on the finished Ortho-R product (100 mg format)-Long-term conditions (25 °C/60 % RH).

CRediT authorship contribution statement

Anik Chevrier: Writing – original draft, Investigation, Formal analysis. **Dong Wang:** Writing – review & editing, Investigation. **Elizabeth Ladd:** Investigation. **Jean-Simon Blais:** Writing – review & editing, Project administration. **Marc Lavertu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marc Lavertu reports financial support was provided by ChitogenX Inc. Marc Lavertu reports financial support was provided by Natural Sciences and Engineering Research Council of Canada. Marc Lavertu reports a relationship with ChitogenX Inc that includes: equity or stocks. Anik Chevrier reports a relationship with ChitogenX Inc that includes: equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2025.100777.

Data availability

Data will be made available on request.

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