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Macromolecular conformation of chitosan in dilute solution: A new global hydrodynamic approach

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ABSTRACT

Chitosans of different molar masses were prepared by storing freshly prepared samples for up to 6 months at either 4, 25 or 40 °C. The weight-average molar masses, M_w and intrinsic viscosities, $[\eta]$ were then measured using size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) and a "rolling ball" viscometer, respectively.

The solution conformation of chitosan was then estimated from:

(a) the Mark–Houwink–Kuhn–Sakurada (MHKS) power law relationship $[\eta] = kM_w^a$ and
(b) the persistence length, L_p calculated from a new approach based on equivalent radii [Ortega, A., & Garcia de la Torre, J. (2007). Equivalent radii and ratios of radii from solution properties as indicators of macromolecular conformation, shape, and flexibility. Biomacromolecules, 8, 2464–2475].

Both the MHKS power law exponent ($a = 0.95 \pm 0.01$) and the persistence length ($L_p = 16 \pm 2$ nm) are consistent with a semi-flexible rod type (or stiff coil) conformation for all 33 chitosans studied. A semi-flexible rod conformation was further supported by the Wales–van Holde ratio, the translational frictional ratio and sedimentation conformation zoning.

1. Introduction

Due to being in the unique position of being the only "natural" polycationic polymer chitosan and its derivatives have received a great deal of attention from the food, cosmetic and pharmaceutical industries. Important applications include water and waste treatment, antitumor, antibacterial and anticoagulant properties (Rinaudo, 2006). The interaction of chitosan with mucus is also important in oral and nasal drug delivery (Harding, Davis, Deacon, & Fiebrig, 1999).

Chitosan is the generic name for a family of strongly polycationic derivatives of poly-*N*-acetyl-D-glucosamine (chitin) extracted from the shells of crustaceans or from the mycelli of fungi (Rinaudo, 2006; Tombs & Harding, 1998). In chitosan (Fig. 1) the *N*-acetyl group is replaced either fully or partially by NH_2 therefore the degree of acetylation can vary from DA = 0 (fully deactylated) to DA = 1 (fully acetylated i.e., chitin).

Chitosan is only soluble at acidic pH (pH < 6) and, therefore, the amine groups exist predominantly in the NH_3^+ form resulting in a highly charged polycationic chain and which is reported to have either a rigid rod type structure (Cölfen, Berth, & Dautzenberg, 2001; Errington, Harding, Vårum, & Illum, 1993; Fee et al., 2003; Kasaai, 2006; Terbojevich, Cosani, Conio, Marsano, & Bianchi, 1991) or a semi-flexible-coil (Berth, Dautzenberg, & Peter, 1998; Brugnerotto, Desbrières, Roberts, & Rinaudo, 2001; Lamarque, Lucas, Viton, & Domard, 2005; Mazeau & Rinaudo, 2004; Rinaudo, Milas, & Le Dung, 1993; Schatz, Viton, Delair, Pichot, & Domard, 2003; Velásquez, Albornoz, & Barrios, 2008; Vold, 2004).

In this paper we will discuss the conformation of chitosan using a recent advancement in the analysis in the molar mass dependencies of intrinsic viscosity and the sedimentation coefficient (Ortega & García de la Torre, 2007).

2. Materials and methods

2.1. Samples

Chitosans (3×) of degree of acetylation (DA) of ~20% were obtained from Pronova Biomedical (Oslo, Norway) and from Sigma Chemical Company (St. Louis, USA) and were used without any further purification. Chitosans (100 mg) were dissolved in 0.2 M, pH 4.3, acetate buffer (100 ml) with stirring for 16 h. The sedimenta-

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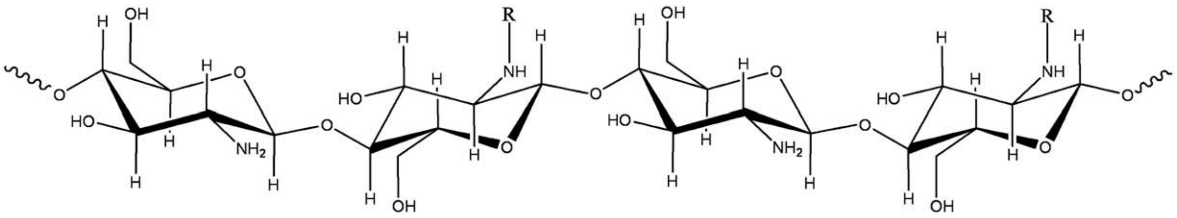

Fig. 1. Schematic representation of the structure repeat units of chitosan, where R = Ac or H depending on the degree of acetylation.

tion coefficient, weight-average molar mass and intrinsic viscosity for each chitosan was measured directly after preparation. Additionally the weight-average molar masses and intrinsic viscosities were measured after the storage of the each of the three chitosan samples for 2 weeks at 25 °C and for 1, 3 and 6 months at either 4, 25 or 40 °C. Resultant chitosans were numbered 1–33 in descending molar mass order.

2.2. Viscometry

The densities and viscosities of samples solutions and reference solvents were analysed using an AMVn Automated Micro Viscometer and DMA 5000 Density Meter (both Anton Paar, Graz, Austria) under precise temperature control (20.00 ± 0.01 °C). The relative, η_{rel} and specific viscosities, η_{sp} were calculated as follows:

$$\eta_{\mathrm{rel}} = \left(\frac{\eta}{\eta_0}\right) \tag{1}$$

$$\eta_{\mathrm{sp}} = \eta_{\mathrm{rel}} - 1 \tag{2}$$

where η is the dynamic viscosity (i.e., corrected for density) of a chitosan solution and η_o is the dynamic viscosity of buffer (1.0299 mPas).

Measurements were made at a single concentration ($\sim 1.0 \times 10^{-3}$ g ml^{-1}) and intrinsic viscosities, $[\eta]$, were estimated using the Solomon–Ciutâ approximation (Solomon & Ciutâ, 1962):

$$[\eta] \approx \frac{\left(2\eta_{\mathrm{sp}} - 2\ln(\eta_{\mathrm{rel}})\right)^{1/2}}{c} \tag{3}$$

2.3. Size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS)

Analytical fractionation was carried out using a series of SEC columns TSK G6000PW, TSK G5000PW and TSK G4000PW protected by a similarly packed guard column (Tosoh Bioscience, Tokyo, Japan) with on-line MALLS (Dawn DSP, Wyatt Technology, Santa Barbara, USA) and refractive index (Optilab rEX, Wyatt Technology, Santa Barbara, USA) detectors. The eluent (0.2 M, pH 4.3, acetate buffer) was pumped at 0.8 ml min^{-1} (PU-1580, Jasco Corporation, Great Dunmow, UK) and the injected volume was 100 µl ($\sim 1.0 \times 10^{-3}$ g ml^{-1}) for each sample. Absolute weight-average molar masses (M_w) were calculated using the ASTRA® (Version 5.1.9.1) software (Wyatt Technology, Santa Barbara, USA), using the refractive index increment, dn/dc = 0.163 ml g^{-1} (Rinaudo et al., 1993).

2.4. Sedimentation velocity in the analytical ultracentrifuge

Sedimentation velocity experiments were performed using a Beckman Instruments (Palo Alto, USA) Optima XLI Analytical Ultracentrifuge. Chitosan solutions (380 µl) of various concentrations (0.1–3.0 mg/ml) and 0.2 M, pH 4.3, acetate buffer (400 µl) were injected into the solution and reference channels, respectively, of a double sector 12 mm optical path length cell. Samples were centrifuged at 45000 rpm at a temperature of 20.0 °C. Concentration profiles and the movement of the sedimenting boundary in the analytical ultracentrifuge cell were recorded using the Rayleigh interference optical system and converted to concentration (in units of fringe displacement relative to the meniscus, j) versus radial position, r (Harding, 2005). The data was then analysed using the "least squares, ls-g(s) model" incorporated into the SEDFIT (Version 9.4b) program (Schuck, 1998, 2005). This software based on the numerical solutions to the Lamm equation follows the changes in the concentration profiles with radial position and time and generates an apparent distribution of sedimentation coefficients in the form of $g^*(s)$ versus $s_{\mathrm{T,b}}$, where the * indicates that the distribution of sedimentation coefficients has not been corrected for diffusion effects (Harding, 2005).

As sedimentation coefficients are temperature and solvent dependent it is conventional to convert sedimentation coefficients (or their distributions) to the standard conditions of 20.0 °C and water using the following equation (Ralston, 1993).

$$s_{20,\mathrm{w}} = s_{\mathrm{T,b}}\left[\frac{(1-\bar{v}\rho_{20,\mathrm{w}})\eta_{\mathrm{T,b}}}{(1-\bar{v}\rho_{\mathrm{T,b}})\eta_{20,\mathrm{w}}}\right] \tag{4}$$

where $\bar{v}$ = 0.57 ml g^{-1} is the partial specific volume of chitosan (Errington et al., 1993) and $\eta_{\mathrm{T,b}}$ and $\rho_{\mathrm{T,b}}$ are the viscosity and density of the experimental solvent (0.2 M, pH 4.3, acetate buffer) at the experimental temperature (20.0 °C) and $\eta_{20,\mathrm{w}}$ and $\rho_{20,\mathrm{w}}$ are the viscosity and density of water at 20.0 °C.

To account for hydrodynamic non-ideality (co-exclusion and backflow effects), the apparent sedimentation coefficients ($s_{20,\mathrm{w}}$) were calculated at each concentration and extrapolated to infinite dilution using the following equation (Gralén, 1944; Ralston, 1993; Rowe, 1977).

$$\frac{1}{s_{20,\mathrm{w}}} = \frac{1}{s^0_{20,\mathrm{w}}}(1 + k_s c) \tag{5}$$

where k_s (ml g^{-1}) is the sedimentation concentration dependence or "Gralén" coefficient (Gralén, 1944).

3. Results and discussion

3.1. Intrinsic viscosity and molar mass

Intrinsic viscosities and weight-average molar masses (Table 1) are in the range 270–1765 ml g^{-1} and 65000–425000 g mol^{-1}, respectively, reflecting depolymerisation of the chitosan chain upon storage at different temperatures for different times.

3.2. Sedimentation coefficient

The sedimentation coefficients (Table 2) were calculated for three chitosans (1, 8 and 25) and reflect the differences in molar mass between the samples.

Table 1
solution properties for chitosan in 0.2 M pH 4.3 acetate buffer.

Sample	$[\eta]$ (ml g^{-1})	M_w (g mol^{-1})
Chitosan-1	1765 ± 55	425000 ± 20000
Chitosan-2	1350 ± 40	400000 ± 15000
Chitosan-3	1530 ± 45	380000 ± 20000
Chitosan-4	1370 ± 40	365000 ± 15000
Chitosan-5	1175 ± 35	340000 ± 5000
Chitosan-6	1210 ± 35	320000 ± 15000
Chitosan-7	1120 ± 35	320000 ± 10000
Chitosan-8	1450 ± 40	290000 ± 20000
Chitosan-9	1180 ± 35	290000 ± 20000
Chitosan-10	1075 ± 30	290000 ± 15000
Chitosan-11	1265 ± 40	275000 ± 20000
Chitosan-12	1125 ± 35	270000 ± 20000
Chitosan-13	1020 ± 30	270000 ± 20000
Chitosan-14	1185 ± 35	260000 ± 20000
Chitosan-15	925 ± 30	235000 ± 20000
Chitosan-16	960 ± 30	230000 ± 20000
Chitosan-17	825 ± 25	225000 ± 5000
Chitosan-18	845 ± 25	205000 ± 20000
Chitosan-19	815 ± 25	195000 ± 5000
Chitosan-20	745 ± 20	175000 ± 5000
Chitosan-21	655 ± 20	160000 ± 5000
Chitosan-22	555 ± 15	130000 ± 5000
Chitosan-23	440 ± 15	130000 ± 5000
Chitosan-24	490 ± 15	115000 ± 5000
Chitosan-25	465 ± 15	115000 ± 5000
Chitosan-26	460 ± 15	115000 ± 5000
Chitosan-27	430 ± 15	105000 ± 5000
Chitosan-28	355 ± 10	105000 ± 5000
Chitosan-29	415 ± 10	100000 ± 5000
Chitosan-30	450 ± 15	95000 ± 5000
Chitosan-31	345 ± 10	75000 ± 5000
Chitosan-32	320 ± 10	70000 ± 5000
Chitosan-33	270 ± 10	65000 ± 5000

Table 2
Hydrodynamic parameters derived from sedimentation velocity.

Sample	$s^0_{20,w}$ (S)	k_s (ml g^{-1})	$k_s/[\eta]$	f/f_0	Zone
Chitosan-1	2.15 ± 0.18	680 ± 40	0.39 ± 0.05	16 ± 2	B/C
Chitosan-8	2.13 ± 0.13	800 ± 100	0.55 ± 0.10	13 ± 1	B/C
Chitosan-25	1.38 ± 0.07	340 ± 30	0.73 ± 0.05	11 ± 1	B

3.3. Conformational analysis

3.3.1. Mark–Houwink–Kuhn–Sakurada exponent "a"

Hydrodynamic results obtained from SEC-MALLS and viscosity measurement were further used to study the gross conformation of chitosan (Harding, Vårum, Stokke, & Smidsrød, 1991), taking advantage of the fact that prolonged storage at different temperatures resulted in different weight-average molar mass, M_w, facilitating the use of the "Mark–Houwink–Kuhn–Sakurada" (MHKS) power law relation linking $[\eta]$ with M_w:

$$[\eta] \propto M_w^a \tag{6}$$

The MHKS exponent (a) is derived using double logarithmic plot of intrinsic viscosities versus molar mass (Fig. 2). In this case we find a value for the exponent, a, of (0.95 ± 0.01) which is indicative of a rigid rod type molecule and is in good agreement with previous estimates: 1.0 (Cölfen et al., 2001); 0.96 ± 0.10 (Fee et al., 2003); 0.90 ± 0.20 (Rinaudo, 2006) and 0.87 ± 0.18 (Kasaai, 2006) the latter two being the average exponent for 6 and 14 different solvent conditions, respectively. This procedure assumes a homologous series for the polymers (i.e., they all have approximately the same conformation type): any departure would reveal itself as non-linearity of the logarithmic plots. The dominance of hydrodynamic interactions between chain segments is taken to render insignificant any contribution to the value of the coefficient though solvent draining effects (Tanford, 1961).

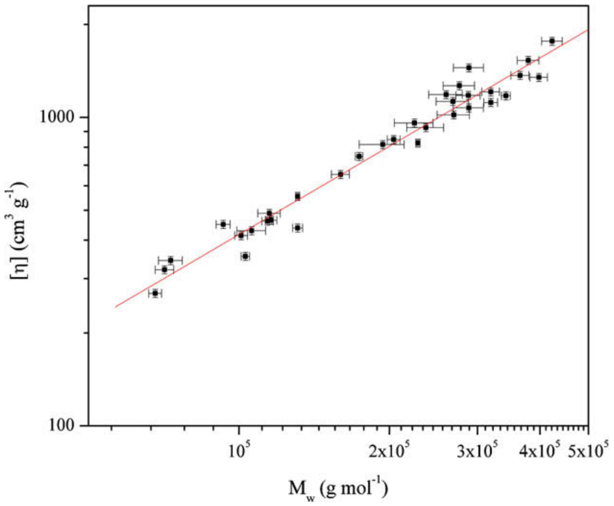

Fig. 2. Mark–Houwink–Kuhn–Sakurada power law double logarithmic plot for chitosan where the slope, $a = 0.95 \pm 0.01$, the intercept log $k = -2.13 \pm 0.05$ and therefore $k = 7.4 \pm 0.9 \times 10^{-3}$ ml g^{-1}.

3.3.2. The translational frictional ratio, f/f$_0$

The translational frictional ratio (Tanford, 1961), f/f_0 is a parameter which depends on molar mass, conformation and molecular expansion through hydration effects. It can be measured experimentally from the sedimentation coefficient and molar mass:

$$\frac{f}{f_0} = \frac{M_w(1-\bar{v}\rho_{20,w})}{(N_A 6\pi\eta_{20,w} s^0_{20,w})}\left(\frac{4\pi N_A}{3\,\bar{v}\,M_w}\right)^{1/3} \tag{7}$$

Values in the range 11–16 (Table 2) are considerably greater than the theoretical minimum of one and could either be due to long chain elongation or a high degree of expansion through (aqueous) solvent association, or a combination of both.

3.3.3. Wales–van Holde ratio, R = k$_s$/[η]

Values for the Wales–van Holde ratio (Wales & van Holde, 1954) in the range 0.39–0.73 (Table 2) are obtained which are similar to those found previously 0.26–0.73 (Cölfen et al., 2001) and are again consistent with extended structures (Morris, Foster, & Harding, 2000, Morris et al., 2008) but short of the limit for rod (0.15) (Harding, Berth, Ball, Mitchell, & Garcìa de la Torre, 1991). It has been previously reported that chitosans of higher molar mass become more compact (Berth et al., 1998) although this is contradicted by the Cölfen et al. (2001) data and also by the new data which both show a decrease in the Wales–van Holde ratio with increase in molar mass, indicating the opposite.

3.3.4. Sedimentation conformation zoning

The sedimentation conformation zone (Pavlov, Harding, & Rowe, 1999; Pavlov, Rowe, & Harding, 1997) plot of log $[s]/M_L$ versus log k_sM_L enables an estimate of the "overall" solution conformation of a macromolecule in solution ranging from Zone A (extra-rigid rod) to Zone E (globular or branched). The parameter $[s]$ related to the sedimentation coefficient by the relation

$$[s] = \frac{s^0_{20,w}\eta_{20,w}}{(1-\bar{v}\rho_{20,w})} \tag{8}$$

and M_L the mass per unit length ≈ 420 g mol^{-1} nm^{-1} (Vold, 2004).

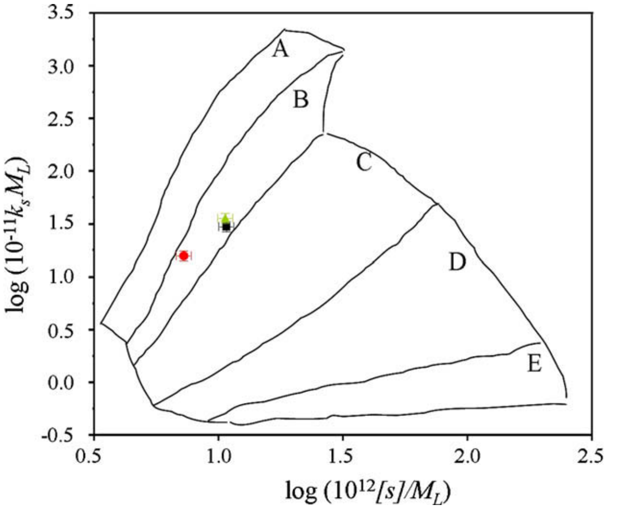

Fig. 3. The sedimentation conformation zoning plot (adapted from Pavlov, Harding, & Rowe, 1999; Pavlov, Rowe, & Harding, 1997). Zone A, extra-rigid rod; Zone B, rigid rod; Zone C, semi-flexible; Zone D, random coil and Zone E, globular or branched. Individual chitosans are marked: chitosan-1 (■), chitosan-8 (▲) and chitosan-25 (●).

The sedimentation conformation zoning (Fig. 3 and Table 2) places all three chitosans as Zone B (rigid rod), although the chitosans 1 and 8 are very close to the boundary with Zone C (semi-flexible coils).

3.3.5. Combined "global" analysis: multi_HYDFIT

The linear flexibility of polymer chains can also be represented in terms of the persistence length, L_p of equivalent *worm-like chains* (Kratky & Porod, 1949) where the persistence length is defined as the average projection length along the initial direction of the polymer chain and for a theoretical perfect random coil $L_p = 0$ and for the equivalent extra-rigid rod (Harding, 1997) $L_p = \infty$, although in practice limits of ~1 nm for random coils (e.g,. pullulan) and 200 nm for an extra-rigid rod (e.g., schizophyllan) are more appropriate (Tombs & Harding, 1998).

The persistence length and mass per unit length can be estimated using the Multi_HYDFIT program (Ortega & García de la Torre, 2007), which considers data sets of intrinsic viscosities and sedimentation coefficients for different molar mass. It then performs a minimisation procedure finding the best values of M_L and L_p and chain diameter d satisfying the Bushin–Bohdanecky (Bohdanecky, 1983; Bushin, Tsvetkov, Lysenko, & Emel'yanov, 1981) and Yamakawa–Fujii (Yamakawa & Fujii, 1973) equations (Eqs. 9 and 10). Extensive simulations have shown that values returned for M_L and L_p are insensitive to d, so this is usually fixed (Ortega & García de la Torre, 2007).

$$\left(\frac{M_w^2}{[\eta]}\right)^{1/3} = A_0 M_L \Phi^{-1/3} + B_0 \Phi^{-1/3}\left(\frac{2L_p}{M_L}\right)^{-1/2} M_w^{1/2} \tag{9}$$

$$s^0 = \frac{M_L(1-\bar{v}\rho_0)}{3\pi\eta_0 N_A} \times \left[1.843\left(\frac{M_w}{2M_L L_p}\right)^{1/2} + A_2 + A_3 + \left(\frac{M_w}{2M_L L_p}\right)^{-1/2} \cdots\right] \tag{10}$$

$$d = \left(\frac{4M_L\bar{v}}{\pi N_A}\right)^{1/2} \tag{11}$$

where $M_L \approx 420$ g mol^{-1} nm^{-1} (Vold, 2004) and the partial specific volume, $\bar{v} = 0.57$ ml g^{-1} (Errington et al., 1993) and therefore d ≈ 0.7 nm.

The Multi_HYDFIT program then floats the variable parameters in order to find a minimum of the multi-sample target (error) function (Ortega & García de la Torre, 2007), Δ. In this procedure as defined in Ortega and García de la Torre (2007), Δ is calculated using equivalent radii, where the equivalent radius (a_x) is defined as the radius of an equivalent sphere having the same value as the determined property. In the present study, we are interested in the equivalent radii resulting from the sedimentation coefficient i.e., translational frictional coefficient (a_T) and from the intrinsic viscosity (a_I).

$$a_T = \frac{f}{6\pi\eta_0} \tag{12}$$

where η_0 is the viscosity of water at 20.0 °C, and

$$a_I = \left(\frac{3[\eta]M_w}{10\pi N_A}\right)^{1/3} \tag{13}$$

where N_A is Avogadro's number.

The target function, Δ can be evaluated from the following relations:

$$\Delta^2 = \frac{1}{N_s}\sum_{i=1}^{N_s}\left[\left(\sum_T W_T\right)^{-1}\sum_T W_T\left(\frac{a_{T(cal)} - a_{T(exp)}}{a_{T(exp)}}\right)^2\right] \tag{14}$$

$$\Delta^2 = \frac{1}{N_s}\sum_{i=1}^{N_s}\left[\left(\sum_I W_I\right)^{-1}\sum_I W_I\left(\frac{a_{I(cal)} - a_{I(exp)}}{a_{I(exp)}}\right)^2\right] \tag{15}$$

where N_s is the number of samples in multi-sample analysis, W_T and W_I are the statistical weights for equivalent radii a_T and a_I (from translation frictional coefficient and intrinsic viscosity data, respectively) and the subscripts cal and exp represent values from calculated and experimental values, respectively.

Δ is thus a dimensionless estimate of the agreement between the theoretical calculated values for the intrinsic viscosity and sedimentation coefficient for a particular molar mass, persistence length and mass per unit length and the experimentally measured parameters (Ortega & García de la Torre, 2007), therefore the value of Δ multiplied by 100% is the percentage difference between theoretical and calculated values.

The minimum in the target function ($\Delta = 0.09$) corresponds to a persistence length of (16 ± 2) nm and a mass per unit length of (450 ± 20) g mol^{-1} nm^{-1} (Fig. 4). If we fix the mass per unit length to 420 nm (Vold, 2004), we find a persistence length of 14 nm. It

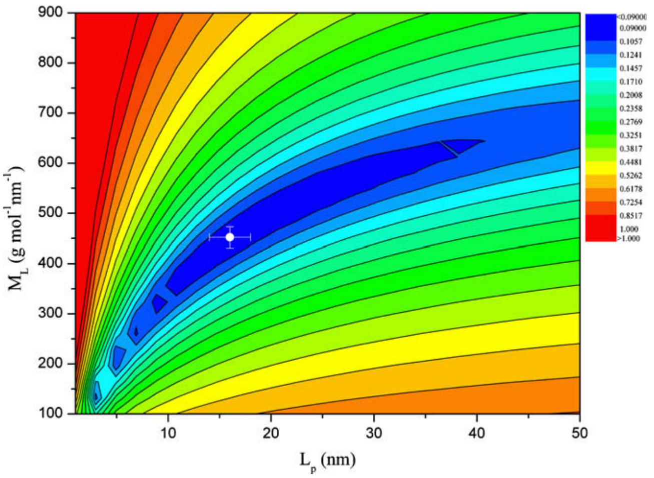

Fig. 4. Solutions to the Bushin–Bohdanecky equations for chitosan using equivalent radii approach. The *x*-axis and *y*-axis represent L_p (nm) and M_L (g mol^{-1} nm^{-1}), respectively. The target function, Δ is calculated over a range of values for M_L and L_p. In these representations, the values of Δ function are represented by the full colour spectrum, from the minimum in the target function in blue ($\Delta = 0.09$) to red ($\Delta \geqslant 1$). The calculated minimum ($L_p = 16 \pm 2$ nm and $M_L = 450 \pm 20$ g mol^{-1} nm^{-1}) is indicated.

Table 3
Persistence length and mass per unit length estimates for chitosan.

Persistence length, L_p (nm)	Mass per unit length, M_L (g mol^{-1} nm^{-1})	Reference
16 ± 2	450 ± 20	This study
22–35	–	Terbojevich et al. (1991)
6–13	340	Berth et al. (1998)
5–13	350	Cölfen et al. (2001)
11–15	–	Brugnerotto et al. (2001)
4–6	–	Schatz et al. (2003)
11–15	–	Mazeau and Rinaudo 2004
5–9	350–470	Vold (2004)
6–15	–	Lamarque et al. (2005)
8–17	–	Velásquez et al. (2008)

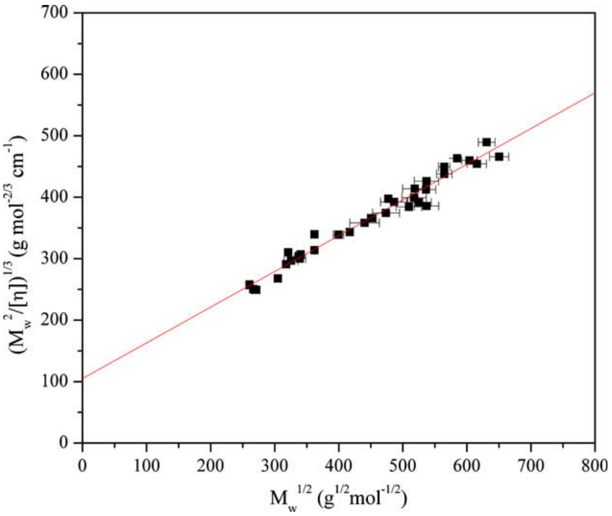

Fig. 5. Bushin–Bohdanecky plot for chitosan where $L_p = 22 \pm 2$ nm from the slope and $M_L = 520 \pm 20$ g mol^{-1} nm^{-1} from the intercept.

should, however, be noted that all values of Δ in the first contour vary by less than the experimental error ~2% and, therefore, we are most likely looking at a spectrum of probable conformations where L_p and M_L range from 5 to 40 nm and 220 to 650 g mol^{-1} nm^{-1}, respectively, which may go some way to explaining why chitosan has been described as either a semi-flexible coil or a rigid rod.

4. Conclusions

Several previous studies on the solution conformation of chitosan (Table 3) (Cölfen et al., 2001; Errington et al., 1993; Fee et al., 2003; Kasaai, 2006; Terbojevich et al., 1991) have suggested a rigid rod conformation whilst others (Berth et al., 1998; Brugnerotto et al., 2001; Lamarque et al., 2005; Mazeau & Rinaudo, 2004; Rinaudo et al., 1993; Schatz et al., 2003; Velásquez et al., 2008; Vold, 2004) have adopted a semi-flexible coil model.

This apparent discrepancy has been in part explained by the new Multi_HYDFIT approach (Ortega & García de la Torre, 2007) which has shown that conformation of chitosan is close to the semi-flexible coil—rigid rod limit and that there are a large number of possible conformations which could fall in to either of these categories (Fig. 4). This observation would not have been possible with the more traditional Bushin–Bohdanecky analysis of plotting $\left(\frac{M_w^2}{[\eta]}\right)^{1/3}$ versus $M_w^{1/2}$ (Fig. 5).

It may therefore be prudent to describe the solution conformation of chitosan as a semi-flexible rod (or stiff coil).

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